The investigation of the cardioprotective properties of metformin during sunitinib-induced cytotoxicity

Kuburas, Refik

Award date:
2020

Awarding institution:
Coventry University

The Investigation of the cardioprotective properties of Metformin during Sunitinib-induced cytotoxicity

By

Refik Kuburas

B.Sc, M.Sc

For the award for Ph.D

October 2019

The Investigation of the cardioprotective properties of Metformin during Sunitinib-induced cytotoxicity

By

Refik Kuburas

A thesis submitted in partial fulfillment of the University's requirements for the Degree of Doctor of Philosophy (Ph.D)

October 2019

Certificate of Ethical Approval

Certificate of Ethical Approval

Applicant:

Refik Kuburas

Project Title:

The Cardioprotective and Anticancer properties of Metformin Treatment in Sunitinib-Induced Cardiotoxicity and Treatment: The Role and Importance of AMPK

This is to certify that the above named applicant has completed the Coventry University Ethical Approval process and their project has been confirmed and approved as Low Risk

Date of approval:

13 May 2016

Project Reference Number:

P43367

Candidates Declaration

Content removed on data protection grounds

Content removed on data protection grounds

Turnitin Report Summary

Content removed on data protection grounds

Acknowledgements

I would like to offer my thanks and gratitude to my supervisors; Dr Hardip Sandhu, Prof. Helen Maddock, Dr Irmgard Haussmann and Dr Mayel Gharanei for their support, expertise and guidance throughout this project. I would also like to thank Dr Hardip Sandhu and Prof Helen Maddock for providing me with the opportunity to undertake and complete this project and ultimately my PhD. I would also like to thank Coventry University and the Faculty of Sport, Exercise and Life Sciences (formerly applied biological and exercise sciences) for funding this project and proving me with the studentship to undertake this project.

I would also like to extend my gratitude to the Faculty of Health and Life Sciences' technicians, lecturers and teaching staff for their support, expertise and cooperation during the undertaking of this project.

Finally, I would like to thank my colleagues, friends and family for continuous support, motivation and encouragement during this project.

Thank you,

Refik Kuburas

Abstract

Activation of the adenosine monophosphate protein kinase (AMPK) signalling pathway is known to result in the inhibition of ATP-consuming anabolic mechanisms in the heart and is associated with prevention of cardiac myocytes loss. The multi-tyrosine kinase inhibitor (TKI) Sunitinib has been associated with cardiotoxicity via the inhibition of AMPK signalling. In contrast, the agent Metformin, used for treatment of type-2 diabetes mellitus, is associated with cardioprotective properties with the activation of the AMPK signalling pathway. We aimed to demonstrate the potential use of Metformin with Sunitinib in order to prevent Sunitinib-induced cardiotoxicity. Sprague-Dawley (2/3 m/o, male) rat hearts were Langendorff perfused with vehicle control, Sunitinib (1 µM) ± Metformin (50 µM) ± AMPK-associated inhibitor S-(4-Nitrobenzyl)-6-thioinosine (NBTI) (1 µM) for 155 minutes, following 20 minutes of stabilisation. Haemodynamic parameters were measured for heart rate (HR), left ventricular developed pressure (LVDP) and coronary flow (CF). Dismounted hearts were used for triphenyltetrazolium chloride (TTC) staining for infarct percentage or Western blot SDS page analysis for protein levels for phosphorylated-AMPK (Thr^{172}), total-AMPKα and GAPDH. Isolated primary cardiac myocytes were obtained from Sprague-Dawley (2/3 m/o, male) rat hearts following Langendorff perfusion. HepG2 and HL60 cells were incubated with Sunitinib (0.1-100 µM) ± Metformin (50 µM) ± NBTI (1 µM) for (3-(4,5-Dimethylthiazol-2-yl)-2-5-Diphenyltetrazolium Bromide (MTT) analysis. Sunitinib administration (1 µM) demonstrated a significant increase in infarct percentage of Sprague-Dawley rat hearts using the Langendorff model, compared to vehicle control. Sunitinib administration further demonstrated a significant decrease in LVDP compared to vehicle control at selected time-points. Co-administration of Sunitinib with Metformin (50 µM) attenuated the increase in infarct percentage (co-treatment 20 ± 2 % vs. Sunitinib 31 ± 2 %) and attenuated the Sunitinib-induced decrease in LVDP at selected time-points (145 minute; Sunitinib 64 ± 5 % vs. co-treatment 80 ± 6 %, 160 minute; Sunitinib 57 ± 6 % vs. co-treatment 75 ± 3 %, 175 minute; Sunitinib 58 ± 6 % vs. co-treatment 74 ± 3 %). Live cell population of cardiac myocytes was decreased during Sunitinib administration (1 µM). Co-administration of Metformin (50 µM) with Sunitinib attenuated Sunitinib-induced decrease of live cell population of isolated cardiac myocytes (co-treatment 41 ± 3 % vs. Sunitinib 12 ± 2 %).

Metformin-induced activation of AMPK and Metformin-induced cardioprotection was abolished during co-administration of Sunitinib and Metformin with NBTI (1 µM). Sunitinib demonstrated a dose-dependent decrease in cell viability in HepG2 and HL60 cells, co-administration of Sunitinib (0.1-100 µM) with Metformin (50 µM) demonstrated an increase in EC_{50} concentration in HepG2 and HL60 cells (HepG2 cells; co-treatment 34.7 µM vs. Sunitinib 15.4 µM., HL60 cells; co-treatment 18.2 µM vs. Sunitinib 10 µM). The addition of NBTI (1 µM) with Sunitinib (0.1-100 µM) and Metformin (50 µM) demonstrated an increase in EC_{50} concentration in HepG2 (43.3 µM) and HL60 cells (33.3 µM).

We demonstrated for the first time Metformin-induced cardioprotection against Sunitinib-induced cardiotoxicity in an *ex-vivo* Langendorff-based model. Furthermore, Western blot analysis determined that Metformin-induced cardioprotection is associated with an increase in phosphorylation of AMPK, an effect that was abolished upon AMPK inhibition with NBTI. Moreover, we demonstrated the adjunctive treatment in human cancer cell lines of HepG2 and HL60, that Metformin co-administration resulted in an increase in EC_{50} concentration compared to Sunitinib administration alone, however Metformin did not inhibit Sunitinib's anti-proliferative properties.

In conclusion, this study demonstrates the cardioprotective properties of Metformin during co-administration with Sunitinib and the role of AMPK signalling. This study adds to the growing interest of potential adjunctive treatment during TKI therapy.

Statement of significance

Early detection of symptoms associated with drug-induced cardiotoxicity can prevent the onset of ischaemia, heart failure as well as drug discontinuation. Screening with human cancer cell lines can reveal if potential adjunctive therapy drugs interfere with and prevent the anti-cancer activity of chemotherapy agents. Sunitinib has been associated with cardiotoxicity with the involvement of the AMPK signalling pathway, whilst Metformin, used for treatment of type-2 diabetes mellitus, is associated with cardioprotective properties with the activation of the AMPK signalling pathway. We demonstrated for the first time the potential use of Metformin with Sunitinib in an *ex-vivo* Langendorff-based model. We demonstrated for the first time in the described model that Metformin protects against Sunitinib-induced cardiotoxicity. Metformin-induced cardioprotection was correlated with an increase in phosphorylated-AMPK signalling following Western blot analysis, an effect that was attenuated following AMPK inhibition. The proposed activation of AMPK acts to counter loss of ATP levels, highlighted by current literature, corresponding to events of ischaemia, and the prevention of the activation of apoptosis and necrosis, preventing the loss of cardiac myocytes and coronary tissue.

Contents from this thesis was submitted / published as following:

Kuburas, R., Gharanei, M., Haussmann, I., Maddock, H., and Sandhu, H. (2019) ‘Metformin Protects Against Sunitinib-Induced Cardiotoxicity via the Activation of AMPK Signalling’. Submitted to British Journal of Pharmacology

Sandhu, H. and Kuburas, R. (2015) 'Insulin Resistance in Women with Polycystic Ovary Syndrome: Optimizing Treatment by Implementing an in Vitro Insulin Resistance Organ Culture Model'*. Clinics Mother Child Health* 12, e107.

Kuburas, R., Gharanei, M., Haussmann, I., Maddock, H., and Sandhu, H. (2018)*. ‘P37 the Assessment of the Cardioprotective Properties of Metformin during Sunitinib-Induced Cytotoxicity’* A14-A14*.*

Kuburas, R., Gharanei, M., Haussmann, I., Maddock, H., and Sandhu, H. (2017) 'The Early Assessment of the Cardioprotective Properties of Metformin during Sunitinib-Induced Cytotoxicity'. *Journal of Pharmacological and Toxicological Methods* 88, 218.

Contents

List of Figures and Tables

Figures

Tables

Abbreviations

%	Percentage
µM	Micromole
4E-BP1	Elongation factor-4E binding protein 1
5’-ITU	5’-iodotubercidin
8-SPT	8-p-sulfophenyltheophylline hydrate
ABL	Abelson
ACC	Acetyl-coenzyme A carboxylase
Acetyl-CoA	Acetyl-coenzyme A
ADP	Adenosine diphosphate
Akt	Protein kinase B
AICAR	Aminoimidazole carboxamide ribonucleotide
AIF	Apoptosis-inducing factor
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate protein kinase
ANT	Adenine nucleotide translocase
Apaf	Apoptosis protease activating factor
ATCC	American Type Culture Centre
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BAD	Bcl2-antagonist of cell death
Bax	Bcl2-associated X protein
BclXL	B cell lymphoma–XL

BCR	Break-point cluster region
BMI	Body mass index
BMP	Bone morphogenic protein
cAMP	Cyclic AMP
CAMKK	Calmodulin-dependent protein kinase
CHD	Coronary heart disease
CF	Coronary flow
cIAP	Cellular inhibitor of apoptosis
CNT	Concentrative nucleoside transporter
CPT	Carnitine palmitoyl transferase
CRTC2	cAMP response element-binding protein-regulated transcription coactivator 2
CSF1R	Colony-stimulating factor 1 receptor
CVD	Cardiovascular disorder
CyPD	Cyclophillin D
DDIT4	DNA-damage-inducible transcript 4
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
ECG	Electrocardiogram
EEF2	Eukaryotic elongation factor-2
EGFR	Epidermal growth factor receptor
ENT	Equilibrative nucleoside transporter

EPC	Endothelial progenitor cell
Erk	Extra-cellular signal-regulated kinase
EIF2α	Eukaryotic translation initiation factor 2α
FADD	FAS-associated death domain protein
FAK	Focal adhesion kinase
FBPase	Fructose-1, 6-biphosphatase
FBS	Fetal bovine serum
FLIP	FLICE-like inhibitory protein
FLT	FMS-like tyrosine kinase-3
FOCO1	forkhead box-O1
GADD45	Growth arrest and DNA damage–inducible 45
GIST	Gastro-intestinal stromal tumours
GTP	Guanosine triphosphate
hENT	Human equilibrative nucleoside transporter
HF	Heart failure
HIF	Hypoxia-inducible factor
HR	Heart rate
IAP	Inhibitors of apoptosis
ICAD	Inhibitor of caspase activated DNase
ICAM	Intercellular adhesion molecule
IGF	Insulin–like growth factor
I_{Kr}	Rapid delayed rectifier potassium current
I_{Ks}	Slow delayed rectifier potassium current

IL	Interleukin
IRE1	Inositol-requiring enzyme 1
IRS	Insulin receptor substrate
JAK	Janus-kinase
JNK	Jun-N-terminal kinase
KH	Krebs Henseleit
LDH	Lactate-dehydrogenase
LV	Left ventricular
LVDP	Left ventricular developed pressure
LVEF	Left ventricular ejection fraction
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
MCT4	Mono carboxylate transporter 4
MI	Myocardial infarction
mg/d	Milligram per day
mg/dl	Milligram per decilitre
mg/kg	Milligram per kilogram
mg/ml	Milligram per milter
MLC	Myeloid cell leukaemia protein
mmHg	Millimetre of mercury
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
MTT	3-(-4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium

	bromide
mTOR	Mammalian transporter of rapamycin
mTORC1	Mammalian transporter of rapamycin complex 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NBTI	S-(4-Nitrobenzyl)-6-thioinosine
NO	Nitric oxide
NFκB	Nuclear factor–κB
NT-proBNP	N-terminal pro-brain natriuretic peptide
OCT1	Organic cation transporter 1
PAT1	Proton-assisted amino acid transporter 1
PBS	Phosphate buffered saline
PDGFR	Platelet-derived growth factor receptor
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC-1α	PPARγ co-activator 1α
PI3K	Phosphatidylinositol-3-OH kinase
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PTEN	Phosphatase and tensin homolog
PTPN11	Tyrosine protein phosphate non-receptor type 11
RAG	Recombination activating gene
ROS	Reactive oxygen species
RSK	Ribosomal S6 kinase
RISK	Reperfusion injury salvation kinase

S6K1	Ribosomal protein S6 kinase 1
SD	Sprague-Dawley
siRNA	Small interfering RNA
SIRT1	NAD(+)-dependent histone/protein deacetylase sirtuin 1
SREBP	Sterol regulatory element-binding protein
STAT	Signal transducer and activator of transcription
T2DM	Type-2 diabetes mellitus
TDP	Torsade de pointe
Thr^{172}	Threonine 172
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TRAIL	TNF related apoptosis-inducing ligand
TSC	tuberous sclerosis complex
TNF	Tumour necrosis factor
TTC	2, 3, 5 triphenyltetrazolium chloride
TTF	Triphenyltetrazolium formazan
ULK-1	Unc-51 like autophagy activating kinase
VCAM	Vascular cell adhesion molecule
VDAC	Voltage–dependent anion channel
VEGFR	Vascular endothelial growth factor receptor
VHL	von Hippel-Lindau
XIAP	X–linked inhibitor of apoptosis protein

Chapter One: General Introduction

1.1. General overview

The experimental work carried out in this thesis aimed to investigate the potential intracellular pathway and molecular markers associated with the cardioprotective effects of Metformin when used in adjunctive therapy with Sunitinib. This was carried out in order to potentially reduce or prevent Sunitinib-induced cardiotoxic effects during an *ex-vivo* Langendorff perfused rat heart model, with a further aim to examine the effects of co-treatment of adjunctive therapy with Metformin in cancer cell models.

The introductory chapter provides general information on the issue of drug-induced heart disease, the cardiotoxic mechanism of Sunitinib, the potential mode of cardioprotection by Metformin and the emphasis on the role of the AMPK pathway in cardiovascular disease conditions.

1.2. Epidemiology of heart disease

The World Health Organisation estimate that over 17.7 million individuals died from cardiovascular disease (CVD) in 2015, whilst 7.4 million died due to coronary heart disease (CHD) and 6.7 million due to stroke (World Health Organisation 2017). Moreover, the British Heart Foundation reported that CVD results in over 25 % of all deaths within the United Kingdom in 2016–2017, with over 80 % dying in England compared to the United Kingdom overall (British Heart Foundation 2018). Furthermore, 7 million individuals are known to be living in the United Kingdom with CVD, with approximately 200,000 reported hospital visits in the UK resulting from myocardial infarction (MI), typically defined as a heart attack caused by the blockade of the coronary artery preventing the heart from receiving oxygenated blood (British Heart Foundation 2018). Moreover, the National Heart, Lung and Blood Institute (2018) highlighted risk factors to include high blood pressure-blood pressure consistently greater than systolic 140 mmHg / diastolic 90 mmHg (National Heart, Lung and Blood Institute 2018), high blood cholesterol–non-high density lipoprotein greater than 130 mg/dl (National Heart, Lung and Blood Institute 2014), smoking, being overweight or obese–BMI greater than 25.0 (Centres for Disease Control and Prevention 2016), low physical activity as well as having a family history of heart disease and preeclampsia during pregnancy (National Heart, Lung and Blood Institute 2017). Diabetes is also a risk factor for heart disease and heart failure (HF) (van Melle *et al*. 2010). The prevalence of patients with diabetes and HF is said to be rising, and in comparison to HF patients without diabetes, patients with diabetes are reported to have a poor prognosis and quality of life (de Boer e*t al*. 2010, MacDonald *et al*. 2008). Patients with type-2 diabetes mellitus (T2DM) are reported to experience worse clinical outcomes following an incidence of MI compared to non-diabetic patients experiencing MI (Haffner *et al*. 1998, Malmberg *et al*. 2000, McGuire *et al*. 2000). Following this, the resulting development of HF and CVD are known to result in the development of myocardial ischaemia, resulting from an inadequate supply of oxygenated blood to the myocardium (Hurst and Lee 2003).

1.3. Pathophysiology of drug-induced cardiotoxicity and ischaemic injury

As well as the discussed causes and contributors towards CVD, HF and MI discussed in Section 1.2., patients undergoing treatment, particularly chemotherapy, have been associated with the development and increased risk of developing cardiovascular adverse effects. Cardiovascular toxicity remains a serious issue in the clinical and pre-clinical phases of drug development, as well as contributing towards the withdrawal of agents post-approval. Safety issues account for approximately one third of all drug discontinuation (Ferri *et al*. 2013). Due to a very high attrition rate during in-man registration phases of development, the U.S federal drugs agency (FDA) approval of new drugs was lower in 2002 than at any other time in the previous decade (Ferri *et al*. 2013).

Cardiotoxic effects may be induced in a predictable dose and time-dependent manner, whilst cardiotoxic agents may promote adverse consequences in patients with existing cardiovascular co-morbidities (Varga *et al*. 2015, Curigliano *et al*. 2016). Certain agents have been associated with the onset of HF and cardiotoxicity, arising from the delay in ventricular depolarisation and repolarisation, resulting in an elevated QTc interval and QRS complex widening (Silke *et al*. 2002). The QT interval is the measuring of the beginning of the QRS complex to the end of the T wave on the electrocardiogram (ECG) surface, this represents a period from the onset of depolarisation to the completion of repolarisation of the ventricular myocardium (BMJ 2016). In summary, the P>Q interval defines the period of atrial depolarisation and ventricular filling, followed by the period of isovolumetric contraction defined as Q>S (BMJ 2016). From this, R>S>T periods results from the ejection period, whilst the T period occurs following isovolumetric and passive ventricular relaxation, the observed peaks are detected using the electrocardiogram (BMJ 2016).

Prolonged or abnormal repolarisation, resulting from QT interval prolongation, long QT syndrome (LQTS) and/or abnormal T or T/U wave morphology, is known to result in the development of the condition known as “torsade de pointe” (TDP) (Yap and Camm 2003). Further TDP prolongation is known to result in ventricular fibrillation and sudden cardiac death and HF (Yap and Camm 2003). Within healthy individuals, QT interval is approximately 400 ms in men and 420 ms in women, QTc

values greater than 450 ms in men and greater than 470 ms in women is classed as ‘prolonged’ whilst QTc values greater than 500 ms are associated with arrhythmias and TDM (Huffman and Stern 2003, Glassman and Bigger 2001). As highlighted, many agents such as anti-arrhythmic agents and many non-cardiac agents are known to result in the prolongation of the QT and the development of TDP (Yap and Camm 2003). Therefore, understanding and identifying agents that have the potential to result in TDP and associated cardiotoxicity is key for patient survival.

One explanation of the effects of drug-induced prolongation of the QTc interval can be hypothesised to be a direct result of the blockade of the delayed rectifier K^+ current on the cardiac membrane, related to ventricular arrhythmia and can cause sudden cardiac arrest (Erbas and Yilmaz 2013). Repolarisation of cardiac myocytes result from the outward movement of K^+ ions, K^+ currents involved in ventricular repolarisation are subtypes of the delayed rectifier current and include the rapid activation of the delayed potassium rectifier channel (I_{Kr}) and the slow activation of the delayed potassium rectifier channel (I_{Ks}) (Yap and Camm 2003, Guo *et al*. 2011). Blocking of either of these potassium currents is said to be associated with the prolongation of action potential (Yap and Camm 2003). It has been mentioned by Yap and Camm (2003) that I_{Kr} is most susceptible to pharmacological influence and that the blockade is associated with pro-arrhythmic effects of prolonged QT interval and detection of T or U wave abnormalities on the surface ECG. Prolongation of repolarisation is associated with subsequent activation of an inward depolarisation current, referred to as an “early after-depolarisation”, promoting triggered activity and importantly when accompanied by the presence of a notably increased dispersion of repolarisation, the resulting action can induce re-entry and TDP (Yap and Camm 2003). It is known that voltage ion-gated channels are responsible for the generation of electrical activity within the human heart, the Na^+, Ca^+ and K^+ channels are described as the most important for determining shape and duration of cardiac action potential (Brown *et al.* 2000). The direct opening of the K^+ channel results in the movement of positively charged ions out of the cell and therefore terminating action potential and repolarising the myocardium (Brown *et al.* 2000). Drug-induced QTc interval prolongation can be detected using ECG, the prolongation is described to result from the prolonged action potential duration

within individual cardiac cells (Brown *et al.* 2000). As mentioned, the result of defective repolarisation can give rise to “early after-depolarisation” within cardiac cells; triggering arrhythmias as well as TDP (Brown *et al.* 2000). The administration of drugs can result in either the increase in influx of ions into cardiac myocytes during action potential or result in the reduction of efflux (Brown *et al.* 2000). Both alterations are known to result in the prolongation of after-depolarisation and the prolongation of the QTc interval (Brown *et al.* 2000).

The proposed effects can contribute towards repolarisation of the myocardium and therefore contribute towards the potential inducing of CVD, including MI, congestive HF and CHD (Ferri *et al.* 2013). The use of *in-vivo* and *ex-vivo* experiments of animal heart models can assist to monitor and predict these effects during preclinical and early stages of drug development, however direct causes of CVD are said to be continuously less reflected during pre-clinical trials and the outcomes are not often recognised until after the chemotherapeutic agent has been approved and used within a large population and long-term clinical outcomes have been investigated (Ferri *et al.* 2013). Drug-induced cardiotoxicity can induce pericardial and endocardial disorders, contributing towards tachycardia (abnormal heart rate) and tachypnea (abnormal rapid breathing) and can contribute towards the development of stroke within patients (Ferri *et al.* 2013, Song *et al.* 2012).

As mentioned, drug-induced cardiotoxicity results in ischaemic stress which exhibits hallmarks of and occurs directly as a result of the activation of apoptosis within cardiac myocytes (Song *et al.* 2012). This was supported by evidence of apoptosis-induced cardiac myocytes located in the region of ischaemia and zone of infarction, compared to fewer apoptosis-induced cardiac myocytes shown in non-infarct myocardium (Krijnen *et al.* 2002). Moreover, the event of ischaemia is known to result in metabolic changes such as the increase in reactive oxygen species (ROS), calcium overload and the depletion of adenosine triphosphate (ATP) availability (Sanada *et al*. 2011, Eltzschig and Eckle 2011). Furthermore, ischaemia-reperfusion injury can target the mitochondria for cell death, this results in defecting the electron transport chain and oxidative phosphorylation and further contributes to the deprivation of energy availability within the myocardium (Murphy and Steenbergen 2008, Camara *et al*. 2010).

1.4. The role of the adenosine monophosphate protein kinase pathway

The perturbations in energy metabolism during pathological states such as ischaemia and cardiac hypertrophy, as described in Section 1.3., is said to result in the activation of the adenosine monophosphate (AMP)-protein kinase (AMPK) (Hardie and Carling 1997). Activation of AMPK inhibits ATP-consuming anabolic pathways to conserve ATP (Arad *et al*. 2007, Dyck and Lopaschuk 2006). During these events, ATP consumption exceeds the production, resulting in an increase in adenosine diphosphate (ADP) (Qi and Young 2015). Adenylate kinase acts to convert two ADPs to AMP and ATP, increasing cytosolic concentration of AMP and resulting in the activation of AMPK via the binding to the regulatory nucleotide binding domain of the AMPKγ subunit (Qi and Young 2015). AMPK is a heterotrimeric serine/threonine kinase responsible for cellular metabolic control and consists of a catalytic α subunit as well as regulatory β and γ subunits (Shaw *et al*. 2004, Jones and Thompson 2009, Hardie 2011, Viollet *et al*. 2010). Phosphorylation occurs at the threonine 172 (Thr^{172}) phosphorylation site within the α subunit, whilst the β subunit contains binding site for myristoylation, phosphorylation and glycogen (Warden *et al*. 2001, Hudson *et al*. 2003, Polekhina *et al*. 2003, Arad *et al*. 2007). AMPK acts to regulate cellular energy homeostasis within cardiac muscles, and other organ systems, by regulating AMP via the phosphorylation of upstream liver kinase B1 (LKB1) and calmodulin-dependent protein kinase kinase (CAMKK) (Heidrich *et al*. 2010, Arad *et al*. 2007). AMPK remains inhibited by high concentrations of ATP at rest during normal aerobic metabolism, due to ATP binding at the γ subunit (Wang *et al*. 2003, Hardie 2011). Depletion of ATP levels, and events such as stress, hypoxia and ischaemia-reperfusion-induced injury can contribute towards an increasing AMP:ATP ratio, enhancing the stimulation of AMPK via the activation of α subunits at Thr^{172} by kinases LKB1 and CAMKKβ (Frederich and Balschi 2002, Dyck and Lopaschuk 2006, Baron *et al*. 2005, Shaw *et al*. 2004, Jones and Thompson 2009, Hardie 2011, Viollet *et al*. 2010). Two isoforms of the α and β subunits, and three γ isoforms exist within the mammalian genome, human α2 and β2 subunits are expressed within the heart and skeletal muscles, mediating AMPK functions for intracellular localisation (Cheung *et al*. 2000, Arad *et al*. 2007). The AMPK pathway is discussed in greater detail from Section 1.8.

1.5. Myocardial cell death

Cardiotoxicity results in cardiac myocyte death and necrotic cell death during events such as ischaemia (Piper *et al*. 1998). Cardiac myocytes require and utilise high amount of ATP for energy-consuming contractile state, during which malfunctioning mitochondria are replaced by newly synthesised organelles required for the production of ATP (Fischer *et al*. 2012). Mitochondria are required for the production of ATP therefore agents that interfere with the physiological myocardial mitochondrial function are known to induce a depletion of the ATP pool and myocardial dysfunction (Varga *et al*. 2015).

During an event such as ischaemia, within mitochondria altered cytosolic Ca^{2+} regulation induces structural fragility and excessive contractile activation results in necrotic cell death via mitochondrial permeability transition pore (mPTP) opening (Inserte *et al*. 2002, Siegmund *et al*. 1994, Bhamra *et al*. 2008). Opening of the mPTP channel mediates cardiac myocyte death via uncoupling oxidative phosphorylation and swelling of the mitochondria (Hausenloy and Yellon 2003). mPTP has further been described as a non-specific pore within the mitochondrial membrane that opens in the first minutes of myocardial reperfusion in response to ATP depletion and oxidative stress (Griffiths and Halestrap 1995, Javadov *et al*. 2003, Kim *et al*. 2006, Ruiz-Meana *et al*. 2006). Mitochondrial Ca^{2+} overload has been shown to result in mPTP opening (Argaud *et al*. 2004), adding to the understanding that mitochondrial Ca^{2+} uptake during reperfusion has an important role in cell death by bringing mitochondria closer to the threshold at which mPTP opening occurs during reperfusion, known as mitochondrial priming (Weiss *et al*. 2003). Following cardiac myocyte, neutrophil and ischaemic zone endothelial cell injury the generation of oxygen free radicals is known to exacerbate membrane damage, calcium loading and releasing inflammatory mediators all contributing towards microvascular obstruction and the ‘no-flow’ phenomenon in the reperfused myocardium (Buja 2005). The “no-flow” phenomenon is defined as the inadequate myocardial perfusion through a segment of the coronary circulation without angiographic evidence of mechanical vessel obstruction; therefore, re-opening of the coronary artery following obstruction may not result in improved tissue perfusion (Kloner *et al*. 1974, Ramjane *et al*. 2008).

1.6. Apoptosis and Necrosis

As mentioned, ischaemic myocardial cell injury results in programmed cell death (apoptosis) and the rapid loss of cellular homeostasis (necrosis) cell death (Hausenloy and Yellon 2013). Apoptosis is pivotal in determining infarct size within *in-vivo* animal models and *ex-vivo* models of ischaemia-reperfusion injury and drug-induced toxicity (Fliss and Gattinger 1996, Bäcklund *et al.* 2004). As summarised in Figure 1, apoptosis has been described as the highly ordered process displaying nuclear fragmentation and condensation, cytoplasmic shrinkage, blebbing of the plasma membrane, as well as exposure of phosphatidylserine (Yuan, Lipinski and Degterev 2003). Following apoptosis, dead cells fragment into membrane-bound apoptotic bodies for phagocytosis via macrophages and surrounding cells without inducing an inflammatory response (Yuan, Lipinski and Degterev 2003). Apoptosis occurs via the loss of oxygen supply and constrained mitochondrial phosphorylation, thereby initiating the loss of the major source of ATP production for energy metabolism (Buja 2005). Initiating anaerobic glycolysis for ATP production accumulates hydrogen ions and lactate, contributing to intracellular acidosis and glycolysis inhibition via mitochondrial fatty acid and residual energy metabolism (Buja 2005). The resulting impairment in contraction in the sarcolemma establishes the milieu for ventricular arrhythmias (Buja 2005). In comparison to apoptosis, necrosis results in the progressive loss of cytoplasmic membrane integrity, the rapid influx of Na^+, Ca^+ and water causes cytoplasmic swelling, mitochondrial cellular fragmentation, release of lysosomal and granular contents into the extracellular space (Bhatia 2003, Barros and Castro 2001). The resulting effects of necrosis is followed by an inflammatory response to the cellular content released and can further result in tissue damage (Barros and Castro 2001).

An event such as ischaemia and/or ischaemia-reperfusion is said to activate anti-apoptotic pro-survival kinase signalling cascades such as phosphatidylinositol-3-OH kinase (PI3K), protein kinase B (Akt) and p42/44 extra-cellular signal-regulated kinases (Erk) 1-2 (Hausenloy and Yellon 2004). All are said to be implemented in cellular survival through recruitment of anti-apoptotic protection pathways (Hausenloy and Yellon 2004). However, the PI3K/Akt signalling cascade phosphorylates pro-apoptotic proteins Bcl2-antagonist of cell death (BAD), Bcl2-

associated X protein (Bax), Bim and p53 as a result of ischaemia-reperfusion injury (Hausenloy and Yellon 2004, Cross *et al*. 2000). Moreover, Erk 1-2 regulates cell proliferation by mediating cellular apoptosis via inhibiting cytochrome-c induced caspase activation (Hausenloy and Yellon 2004). As highlighted in Figure 1, the Bax protein is known to accelerate apoptosis via inducing mitochondrial cytochrome-c release, resulting in mPTP formation, thus initiating cellular apoptosis (Yamaguchi and Wang 2001, Tsuruta *et al*. 2002, Marzo *et al*. 1998, Hausenloy and Yellon 2004). The Bcl-2 and apoptosis protease activating factor (Apaf) family proteins are said to constitute the core apoptotic mechanism. During cell death the pro-apoptotic Bcl-2 family members: tBid, Dp-5, Bim, Bax, Bak and BAD antagonise anti-apoptotic Bcl-2 family proteins in order to induce mitochondrial damage (Yuan, Lipinski and Degterev 2003). The resulting release of cytochrome-c from the damaged mitochondria is known to recruit caspase-9 and Apaf-1, resulting in the formation of an apoptosome (Yuan, Lipinski and Degterev 2003). The active caspase-9 acts to cleave and activate caspase-3, which further cleaves cellular substrates inhibitor of caspase activated DNase (ICAD) and initiating ICAD-induction of DNA laddering, whilst the release of apoptosis-inducing factor (AIF) and EndoG to facilitate DNA fragmentation and Smac/Diablo to facilitate caspase activation resulting in DNA damage (Yuan, Lipinski and Degterev 2003). Apoptosis is known to be an energy consuming process, and if the energy demand exceeds supply cells are often defaulted to necrotic death (Yuan, Lipinski and Degterev 2003, Ankarcrona *et al*. 1995). The cell deletion process of apoptosis has a pivotal role in cardiovascular diseases including MI, reperfusion injury and the development of HF (Bae *et al*. 2010, Kang and Izumo 2003, Logue *et al*. 2005). Within the mitochondria, AIF and cytochrome-c play an important role in cell viability, however upon release both can additionally activate apoptosis as described in Figure 1 (Bae *et al*. 2010).

Figure 1: Apoptotic pathway in mammalian cells. Activation of apoptosis results in pro-apoptotic Bcl-2 family member proteins: tBid, Dp-5. Bax, Bak, and BAD antagonise Bcl-2 family proteins to induce mitochondrial damage. Following mitochondrial damage, cytochrome-c is released and recruits apoptosome-forming proteins caspase-9 and Apaf-1. Active caspase-9 cleaves and activates caspase-3 resulting in the cleaving of ICAD which initiates ICAD-induction of DNA laddering. This further releases AIF and EndoG and initiates DNA fragmentation following Smac/Diablo activation for caspase activation and DNA damage (Yuan, Lipinski and Degterev 2003).

As described, AIF has an essential down-stream signalling effect in cell death (Bae *et al*. 2010). The nuclear enzyme peroxisome proliferator-activated receptor (PPAR)-1 has been shown to facilitate both the release of AIF from mitochondria and AIF nuclear translocation, as evidenced by PPAR-1 inhibition in the attenuation of Doxorubicin-induced AIF release from mitochondria (Piper *et al*. 1999, Bae *et al*. 2010). Caspases play an integral role in apoptotic cell death (Alnemri *et al*. 1996). Upon mitochondrial damage, cytochrome-c is released and forms an oligomeric complex with dATP or apoptotic proteases, resulting in the activation of Apaf-1 and caspases-9, -3, -6 and -7 (Earnshaw *et al*. 1999, Li *et al*. 1997,). Caspase-8 however is shown to be activated via plasma membrane receptors: Fas receptor and tumour necrosis factor (TNF) receptor, also activating downstream caspases -3 and -7 (Earnshaw *et al*. 1999).

During apoptosis, caspases become cleaved from the existing zymogens form, rendering them active and are capable of permitting caspase-dependent apoptosis; studies have demonstrated the importance of regulating Bcl-2 family proteins and caspase-3 for myocardial protection (Kim *et al*. 2008, Liu *et al*. 2011). The mitochondrial outer membrane permeabilisation has been described to initiate the caspase-dependent apoptotic cascade and caspase-3 was shown to be a pivotal caspase in the mechanism leading to apoptotic death (Earnshaw *et al*. 1999, Li *et al*. 1997). Further to this, ischaemic injury leads to the increase of calcium ions and oxidative stress, resulting in rapid ATP depletion and rupturing the outer mitochondrial membrane, thus initiating apoptosis (Hausenloy *et al.* 2002, Halestrap and Pasdois 2009).

1.7. Multi-tyrosine kinase inhibitors and Sunitinib-induced cytotoxicity

As mentioned in Sections 1.2.–1.5., certain agents have been associated with the onset of HF and drug-induced apoptosis and necrosis of myocardial tissue stemming from cardiac myocyte cell death. An area of particular interest is involving the use of chemotherapeutic agents such as the tyrosine kinase (TK) inhibitors (TKI). A summary of chemotherapy-induced apoptosis is presented in Figure 2. It has been described by Schlessinger (2000) how under normal conditions and disease states such as cancer, TKs are required to modulate proliferation, metabolism, differentiation and apoptosis. Receptor TKs remain active via mutation or translocation and are the main drivers of cancer cell proliferation and survival, whilst TKIs act to catalyse the transfer of phosphate from ATP on a protein substrate, from this the kinases act as a mediator of cellular signal transduction (Gschwind *et al*. 2004). Following this, kinases have become a key therapeutic target within oncology.

From this, phosphorylation of the insulin receptor, epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR) are essential for cellular homeostasis and the modulation of gene expression in intercellular and intracellular signalling pathways involved in cancer development, as highlighted in Figure 3 (Grimminger *et al*. 2010).

Figure 2: Chemotherapy-induced apoptosis. Key: green = anti-apoptotic proteins, red = pro-apoptotic proteins. During treatment, apoptosis can be activated via the intrinsic or extrinsic pathway. Bcl-2 family proteins and the mitochondria are known to regulate the intrinsic pathway, whilst the binding of pro-apoptotic ligands such as TNF related apoptosis-inducing ligand (TRAIL) or Fas act to regulate the extrinsic pathway. Upon activation of apoptosis by chemotherapeutic agents and TKIs, the caspase activated cascade occurs and can act on both extrinsic and intrinsic pathways. The extrinsic pathway results from the binding of pro-apoptotic ligands to cell surface death receptors of the TNF receptor family present on the cell surface or are secreted into the extracellular space, described to belong to the extended cytokine TNF superfamily. Binding results in each receptor forming a death-inducing signalling complex (DISC), recruiting the adaptor protein FAS-associated death domain protein (FADD), caspase-8 and caspase-10. Release of both caspase-8

and caspase-10 into the cytoplasm initiates a subsequent activation of effector caspases to activate apoptosis. The FLICE-like inhibitory protein (FLIP) is known to act to suppress caspase-8 activation. The intrinsic pathway is regulated by Bcl-2 family proteins and mitochondria and is activated via stresses such as DNA damage. Upon activation, p53 transmits death signals via inducing the expression of BH3-only proteins, for the activation of Bax and Bak, resulting in the formation of the mitochondrial outer membrane permeabilisation and releasing mitochondrial apoptogenic proteins; cytochrome-c, SMAC/Diablo and AIF. Cytochrome-c is said to promote the formation of the apoptosome and the activation of caspase-9 to antagonise the inhibitors of apoptosis (IAP) family proteins, whilst AIF and EndoG promote DNA degradation (Zhang and Yu 2013, Ashkenazi 2008, Bagnoli et al. 2010).

TKs of the VEGF receptor are a prime target for the inhibition of angiogenesis for established treatment for the inhibition of tumour growth and metastatic progression of cancer (Figure 3). Treatment options include pyrimidines (e.g. Imatinib, Dasatinib, Nilotinib and Pazopanib), pyrroles (e.g. Sunitinib) and pyridines (e.g. Sorafenib), however therapy is associated with adverse effects such as TDP, hypertension, cardiac toxicity and renal toxicity (Kappers *et al*. 2010). TKs are also involved in the development of certain cancer types. One example of clear-cell renal cell carcinoma involves the inactivation of the von Hippel-Lindau (VHL) gene during VHL disease, resulting in the accumulation of hypoxia-inducible factor (HIF)-1, causing the expression of hypoxia-inducible genes including VEGF and PDGF (Cockman *et al*. 2000, Kaelin Jr 2004, Kim and Kaelin 2004). VHL disease is known to affect several parts of the body, including the development of renal and pancreatic tumours (Oxford University Hospitals 2014). Binding of growth factors to their respective TK receptors, results in cell migration, proliferation and survival, whilst overexpression of VEGF and PDGF is implicated in tumour angiogenesis, growth and metastasis (Krause and Van Etten 2005). Tumour development arises from several pathways and therefore multi-targeted inhibition is considered an effective approach for anti-angiogenic renal cell carcinoma therapy when compared with single-targeted therapy (Oudard *et al*. 2005). However, as summarised in Figure 4, receptor TK signalling is involved in multiple pathways such as the focal adhesion kinase (FAK), PI3K and mitogen-activated protein kinase (MAPK) are involved in non-cancerous cells such as cardiac myocytes.

Figure 3: Signalling pathway of receptor TKs involved in cancer. Ligand binding to the extracellular domain of the receptor TKs results in dimerisation, autophosphorylation and activation of the intracellular dimerisation kinase domain. This results in the recruitment of enzymes, such as PI3K and signal transducer and activator of transcription 3 (STAT3), as well as adaptor molecules and the activation of downstream signalling pathways Akt and Janus-kinase (JAK)-STAT. The enzymes act to regulate transcription, translation, metabolism, cell-proliferation, mortality and survival. Genetic mutations or an overexpression of the described effects, are said to cause malignant proliferation disorders and cancer (Grimminger *et al*. 2010, Gschwind *et al*. 2004, Schlessinger 2000).

Figure 4: Receptor TK signalling in cardiac myocytes. It has been described that within cardiac myocytes, receptor TK signalling is involved in the hypertrophic response to stretch. Integrin signalling acts to activate FAK and SRC, thereby activating PI3K and MAPK signalling cascades following stress (Grimminger *et al*. 2010). MAPK is also said to be activated via transactivation of EGFR by G protein-coupled receptor. Moreover, cytokines released during biochemical stress is said to activate the JAK-STAT pathway. The signalling pathways act to suppress apoptosis, increase protein translocation and transcription of hypertrophy response genes (Grimminger *et al*. 2010, Mendez *et al*. 2005, Ruwhof and van der Laarse 2000).

The TKI Sunitinib (Sunitinib malate, SUTENT, SU11248, Pfizer) is described as a multi-targeted TKI that is currently approved in the United States and European Union (Pfizer 2014). Sunitinib was designed to treat pancreatic cancer, gastrointestinal stromal tumours and renal cell carcinoma, after disease progression or intolerance to Imatinib, by inhibiting VEGF 1-3, platelet-derived growth factor receptor α and β, c-KIT, FMS-like tyrosine kinase (FLT)-3, colony-stimulating factor 1 receptor and RET kinase (Chow and Eckhardt 2007). Furthermore, in 2006 Sunitinib received accelerated approval for the treatment of renal cell carcinoma as well as approval for gastrointestinal stromal tumours (GIST), it is noted that the accelerated approval regulation allows for the approval of drugs used to treat life-threatening illnesses based upon a surrogate end-point considered to be 'reasonably likely to predict clinical benefit' if the drug is an improvement compared to existing therapy (Goodman *et al.* 2007). GIST patients who developed disease progression during Imatinib treatment or patients who were intolerant to Imatinib, no standard treatment was available prior to Sunitinib's production (Goodman e*t al*. 2007).

Sunitinib has been reported to demonstrate clinical activity in solid tumours including breast, neuroendocrine, colorectal, thyroid, melanoma and non-small-cell lung cancer (Chow and Eckhardt 2007). Sunitinib has been able to prolong survival within patients with certain carcinomas including renal cell carcinoma and gastrointestinal stromal tumour after disease progression or intolerance to Imatinib (Motzer *et al*. 2006, Demetri *et al*. 2006, Faivre *et al*. 2006). Sunitinib's further mode of action is known to involve targeting foetal liver TK receptor 3, PDGRF α and β, c-KIT stem cell receptors, *ret* oncogene product colony-stimulating factor 1 receptor (CSF1R) and RET receptor TKs (Branca 2005, Jain *et al*. 2006, Mendel *et al*. 2003, Morabito *et al*. 2006, Faivre *et al*. 2006, Abrams *et al*. 2003).

In-vitro metabolism studies demonstrated that Sunitinib is metabolised via cytochrome CYP3A4 and results in the formation of the *N*-desethyl metabolite SU012662, shown to be potent towards VEGFR, PDGFR and KIT (Baratte *et al*. 2004). SU012662 was concluded to be the major plasma metabolite in mice, rats and monkeys in *in-vivo* experiments, whilst the *N*-oxide metabolite SU012487 was concluded to be the major metabolite in dogs; however, this was said to be infrequently observed in humans (Faivre *et al*. 2006). Radio-labelling of orally

administered Sunitinib demonstrated that primary excretion was over 71 % for rats and 84 % for monkeys (Faivre *et al*. 2006). Animal studies have indicated that target plasma concentrations of Sunitinib and SU012662 were capable of inhibiting PDGFR-*β* and VEGFR-2 phosphorylation at concentrations 50–100 ng/ml (Abrams *et al*. 2003, Mendel *et al*. 2003, Murray *et al*. 2003). These results were said to be similar to those observed in Sunitinib-administered patients with acute myeloid leukaemia, of which sustained inhibition of FLT3 phosphorylation in blast cells was observed (O'Farrell *et al*. 2003).

The 4-week-on, 2-week-off schedule of Sunitinib administration was said to allow patients to recover from potential bone marrow and adrenal toxicity, which was observed in animal models (Faivre *et al*. 2006). Following Sunitinib standard dosage of 50 mg/day for 4 weeks with 2 weeks off-treatment, peak plasma concentration is reached within 6–12 hours (Pfizer 2012). Sunitinib is known to demonstrate linear pharmacokinetics whilst having a long half-life of 40–60 hours (Minkin *et al*. 2009). Following pharmacokinetic analysis, it has been described that dose adjustments are not required for weight, age, gender, race, creatine clearance or Eastern Cooperative Oncology Group Performance Status (EMA nd). The dose range of 50–150 mg/d (15-59 mg/m^2) was assessed in 28 patients with advanced malignancies, for 4 weeks every 6 weeks in the phase I study by Faivre *et al*. (2006), and reported dose-limiting toxicities at doses greater than 75 mg/d, to include reversible grade-3 fatigue, grade-3 hypertension and grade-2 bullous skin toxicity, indicating the recommended dose of 50 mg/d. At this selected dose, manageable toxicities were reported to include sore mouth, edema and thrombocytopenia (Faivre *et al.* 2006).

Following this, the current dosage of 50 mg once-daily of Sunitinib was assessed for efficacy and safety in patients with cytokine-refractory renal cell carcinoma in two phase II trials (Motzer *et al*. 2006, Motzer *et al*. 2007). The first phase II trial involved 63 patients, where 25 patients achieved a partial response in solid tumours, thereby resulting in an objective response rate of 40 %, whilst a further 17 patients achieved a stable disease state over 3 months (Motzer *et al*. 2006). The second phase II trial involved 105 patients, where 33 % obtained a partial response (Motzer *et al*. 2007 (1)). At plasma concentrations of 50-100 ng/ml Sunitinib is able to inhibit the phosphorylation of multiple receptor TKs in tumours, whilst also inhibiting tumour growth, inhibiting metastases and tumour regression in

experimental models of cancer, however it has been highlighted that no minimally effective dose has been established for Sunitinib (Goodman e*t al*. 2007).

The non-receptor TK FAK is believed to be involved in the pathogenesis of cardiac remodelling and hypertrophy via the mediating the first phase of the hypertrophic response to stretch in cardiac myocytes (Torsoni *et al*. 2003). FAK is thought to regulate the activation of myocyte enhancer factor 2 and JNK-Jun pathways in the early activation of the hypertrophic genetic programme (Nadruz *et al*. 2005). The tyrosine protein phosphate non-receptor type 11 (PTPN11) is thought to negatively regulate the growth of cardiac myocytes via downregulation of the mammalian transporter of rapamycin (mTOR) pathway (Marin *et al*. 2008). Nevertheless, cardiovascular events and cognitive HF were still demonstrated with GIST patients receiving Sunitinib (Chu *et al*. 2007). Further studies reported adverse effects on cardiac function and the development of HF with Sunitinib treatment (Khakoo *et al*. 2008, Telli *et al*. 2008).

It has been highlighted that a significant number of patients developed cardiotoxicity following Sunitinib administration, demonstrating congestive HF and electrocardiographic abnormalities (Chu e*t al*. 2007, Hall e*t al*. 2013, Telli *et al*. 2008). Patients were recorded as not experiencing symptoms of cardiotoxicity until after 2 years of treatment with Sunitinib treatment, however approximately 20% of clinical patients experienced significant declines in LV ejection fraction, whilst 47 % of patients were recorded as experiencing hypertension and 8 % developed HF (Chu *et al*. 2007, Motzer *et al*. 2007, Henderson *et al*. 2013). Sunitinib has also been associated with a 1.4 % risk of arterial thrombosis and a 2.69-fold increase in congestive HF (Choueiri *et al*. 2010, Zamorano *et al*. 2016, Motzer *et al*. 2013, Qi *et al*. 2014, Ghatalia *et al*. 2015). Furthermore, Sunitinib-induced cardiotoxicity was found to be more prevalent in patients with a history of heart disease, hypertension or previous treatment with other cardiotoxic agents such as Imatinib, this is particularly concerning as Sunitinib is prescribed following prior-resistance to Imatinib treatment (Henderson *et al*. 2013).

Within animal models Sunitinib upregulated the expression of hypoxia-induced cardiac prolyl-hydroxylase domain-containing protein which is an upstream regulator of HIF-1α, continuous activation of HIF contributes towards congestive HF

(Eschenhagen *et al*. 2011, Moslehi *et al*. 2011). Sunitinib has the potential to activate apoptosis via the release of pro-apoptotic proteins such as cytochrome-c, in doing so it has been reported that Sunitinib inhibits AMPK, with overexpression of an activated mutant AMPK ameliorates Sunitinib-induced cardiac myocyte cytotoxicity (Kerkela *et al*. 2009). Moreover, the study by Laderoute *et al*. (2010) demonstrated Sunitinib directly binding to and inhibiting AMPK phosphorylation. This further reinforces the importance of investigating the direct role of AMPK involvement, with the possibility of designing adjunctive therapy in order to maintain the activation of AMPK.

As summarised in Figure 5, the inactivation of ribosomal S6 kinase (RSK) by Sunitinib is thought to lead to the release of the pro-apoptotic factor BAD (Force *et al*. 2007). This leads to the activation of Bax and cytochrome-c release, further resulting in the activation of the intrinsic apoptotic pathway and ATP depletion as highlighted by Force *et al*. (2007). It was also mentioned by Force *et al*. (2007) that within the setting of energy deficiency or loss of ATP, AMPK would inhibit the energy consuming process during protein translation and lipid biosynthesis via inhibition of eukaryotic elongation factor-2 (EEF2), mTOR and acetyl-coenzyme A (acetyl-CoA) carboxylase (ACC). The inhibition of ACC results in the reduction in malonyl-CoA levels and thereby inhibits carnitine palmitoyltranserase-1 which is responsible for the stimulation of fatty acid oxidation by controlling the entry of long-chain fatty acid molecules into the mitochondria (Dyck e*t al*. 1999, Kudo e*t al*. 1996, Musi e*t al*. 2005, Saddik and Lopaschuk 1991). Inactivation of AMPK by Sunitinib results in the release of EEF2, mTOR and ACC from inhibition; this further exacerbates ATP depletion (Force *et al*. 2007). The study by Laderoute *et al.* (2010) further investigated the direct role of Sunitinib on AMPK inactivation. Laderoute *et al.* demonstrated the ability for sunitinib to directly bind to and inhibit AMPK phosphorylation in PC3 human prostate cancer cells when incubated at concentrations of 5 μM and 20 μM for 6 hours. The effects were said to be similar to that of Compound C. It was proposed by Laderoute *et al.* (2010) that Sunitinib binds via a structurally-dependent basis to the ATP site of the AMPKα catalytic domain. Moreover, as AMPK signalling is considered vital in response to cells in hypoxic conditions, cardiac myocyte survival may be affected by Sunitinib within settings of which hypoxia occurs, notably during ischaemia. The increased activity

of EEF2 and mTOR, in the presence of Sunitinib, would promote hypertrophy, which is further triggered via cardiac myocyte loss and left ventricular (LV) dysfunction (Force *et al*. 2007).

Figure 5: The potential mechanism of action of Sunitinib-induced cardiotoxicity involving the release of BAD, Bax, cytochrome-c, and the inhibition of AMPK activation as summarised by Force *et al*. (2007). Sunitinib is mentioned to result in the inactivation of RSK signalling, resulting in the release of pro-apoptotic proteins BAD, Bax as well as cytochrome c release. As mentioned, the proposed inhibition of AMPK signalling via Sunitinib is hypothesised to result in the release of EEF2, mTOR and ACC from inhibition and further exacerbate ATP depletion (Force *et al*. 2007).

1.8. Sunitinib treatment-associated cardiovascular effects and potential adjunctive treatment, the role of AMPK and mTOR signalling

It was estimated that 2.5 million people 2015 were living with cancer in the UK, it has been reported that the number of people who have survived 5 years or more since diagnosis has increased by 260,000 and the number of projected survivors in the UK is estimated to increase by 1 million per decade from 2010–2040 (Maddams *et al*. 2012, Macmillan Statistics Fact Sheet 2017). Chemotherapeutic agents are known to contribute towards cardiotoxicity, particularly leading to HF, MI, arrhythmias, hypertension and thromboembolism (Bovelli *et al*. 2010). Chemotherapy treatment has been suggested to contribute towards CV damage via direct damage caused by the treatment or via contributing towards accelerated atherosclerosis due to chemotherapy-related CVD risk factors (De Haas *et al*. 2011, Aleman *et al*. 2014).

Improvements in treatment and earlier diagnosis have both contributed towards this increased survival rate for patients, however chemotherapy carries the risk of late effects including CVD that can lead to significant mortality and morbidity accounting for 30–50 % of all deaths within developed countries (Borchmann *et al*. 2012, Clarke *et al*. 2005, Darby *et al*. 2011, Aleman *et al*. 2014, Hodgson 2011). It is therefore crucial to detect cardiac injury at an early onset, thereby preventing further damage. It was mentioned by Force *et al*. (2007), the perspective of cancer treatment, the targeting of multiple TKs is described to make sense, however some agents have an increased risk of cardiotoxicity than more selective agents such as Imatinib.

The selective TKI Imatinib mesylate (Gleevec), used for the treatment of chronic myeloid leukaemia, is associated with cardiotoxic events and there have been reported incidences of peripheral edema (Kerkela *et al*. 2006). Subclinical cardiovascular damage was demonstrated in patients receiving Imatinib, developing congestive HF and LV dysfunction, with similar effects demonstrated in mice following Imatinib treatment (Kerkela *et al*. 2007). Moreover, drug-induced toxicity may arise years after the end drug usage (Ewer and Ewer 2010), Maharsy *et al*. (2014) demonstrated the potential for Imatinib to cause a deleterious effect in cardiac myocytes in a time, dose and age dependent manner, highlighting the

importance in demonstrating the effects of drug-induced toxicity in a time-dependent and long–term manner.

Imatinib acts to inhibit kinase activity of the oncogenic fusion protein break-point cluster (BCR)-abelson (ABL) BCR-ABL and shows marked effects in individuals with chronic-phase chronic myeloid leukaemia (Deininger *et al*. 2005). When investigated with cultured cardiac myocytes, and in mice involving the reproducible doses equivalent to that of a plasma concentration within humans, Imatinib demonstrated mitochondrial dysfunction with a loss of membrane potential, release of cytochrome-c into the cytosol, a reduction in ATP content and cell death (Kerkela *et al*. 2006, Force *et al*. 2007). However, the 5-year follow-up study by Druker *et al*. (2006) involving 553 patients with chronic myeloid leukaemia receiving Imatinib treatment, only a single patient was recorded to have developed drug-related coronary HF (Druker *et al*. 2006). Supporting this, a 6-year follow-up study involving patients with GIST reported no cardiac events attributed to Imatinib treatment (Hochhaus *et al*. 2008). Studies involving cardiac myocytes demonstrated that Imatinib increased calcium-opening of the mPTP in mitochondria isolated from rat hearts and caused significant mitochondrial dysfunction with a loss of membrane potential, declines in ATP and release of cytochrome-c, all associated with cell death via apoptosis (Figure 6) (Dyck and Lopaschuk 2006, Yuan *et al*. 2003, Ankarcrona *et al*. 1995). As cardiac myocytes require large amounts of ATP, due to their contractile role, they are known to be highly sensitive to perturbations in energy generation (Dyck and Lopaschuk 2006). The opening of mPTP plays a significant role in myocardial injury and therefore blocking the opening of the pore should result in cardioprotection, interestingly however the mPTP is said to remain closed during ischaemia and opens at the onset of reperfusion (Suleiman *et al*. 2001, Weiss *et al*. 2003, Griffiths and Halestrap 1995).

Figure 6: The role of Imatinib in cardiac myocyte-activation of apoptosis. The BCR gene and ABL gene kinase inhibitor Imatinib is described to induce extracellular-regulated stress by activating protein kinase RNA-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1 (IRE1) pathways, whilst over expressing protein kinase C (PKC)-delta (PKCδ). It has been described that following sustained extracellular stress, IRE1 activates Jun-N-terminal kinases (JNKs) and as part of the protective response PERK phosphorylates the eukaryotic translation initiation factor 2α (EIF2α), all contributing towards the release of Bax and mitochondrial depolarisation, ATP depletion, cytochrome-c release and further potential features of necrotic and apoptotic cell death (Force *et al*. 2007).

Following Imatinib resistance in patients undergoing treatment for chronic myeloid leukaemia, the second generation multi-TKI Sunitinib malate was produced (Liang *et al*. 2013). Imatinib resistance is reported to develop from a failure to reach complete haematological response and major molecular response, equivalent to a reduction in BCR-ABL transcripts less than 0.1 %, within an allocated duration of time (Milojkovic and Apperley 2009). It was reported that a loss of previously obtained response to Imatinib occurs in 20–25 % of patients that reach a complete haematological response (Milojkovic and Apperley 2009). Sunitinib is known to result in the inhibition of several off-target kinases within patients; this was demonstrated involving the testing of the binding affinity of several small-molecule inhibitors to 119 tyrosine and serine/threonine kinases (Fabian *et al*. 2005). Sunitinib was shown to inhibit normal variants or tyrosine kinases among non-cancerous cells, despite being designed to inhibit kinases that are mutated or over-expressed in cancer cells (Chu *et al.* 2007). Clinical studies in patients treated for gastrointestinal stromal tumours or renal cell carcinoma demonstrated evidence towards Sunitinib-associated cardiotoxicity via HF, LV systolic dysfunction and hypertension (Chu *et al*. 2007, Telli *et al*. 2008).

The phase III trial involving Sunitinib vs interferon-α in patients with previously untreated metastatic renal cell carcinoma, reported that Sunitinib was associated with a 2 % grade-3 decline in LV ejection fraction (LVEF), in comparison to a 1 % grade-3 decline during treatment with interferon-α, this cardiac dysfunction was said to be reversible upon discontinuation or dose lowering of Sunitinib (Motzer *et al*. 2007). LV dysfunction is defined as an absolute decline in LVEF greater than 10 % to a resultant value of less than 50 % (Narayan *et al*. 2017, Cardinale *et al*. 2006, Piccart-Gebhart *et al*. 2005). The study by Narayan *et al*. (2017) reported that patients undergoing Sunitinib treatment for multiple renal cell carcinoma demonstrated “very modest” declines in LVEF, whilst approximately 10 % of patients demonstrated a more substantial decline in LV function, however recovery was demonstrated. However, the report by Catino *et al*. (2018) reported that no statistically significant association was shown between vascular and systolic dysfunction and LVEF, during Sunitinib treatment in patients with metastatic renal cell carcinoma. Moreover, the authors concluded that Sunitinib did result in early, significant increases in blood pressure, arterial stiffness and resistive and pulsative

load during treatment. Sunitinib was further demonstrated to induce apoptosis of human umbilical vein endothelial cells in *in-vitro* and adrenal toxicity in rats, and bone marrow depletion affecting the pancreas in rats and monkeys (Mendel *et al*. 2003, Faivre *et al*. 2006). It was shown in monkeys that Sunitinib resulted in an increase in arterial blood pressure and QT interval at doses that were deemed high (Mendel *et al*. 2003, Faivre *et al*. 2006).

The study by Chu *et al*. (2007) focussed on cardiac adverse effects in 75 patients with Sunitinib treatment and indicated the potential for cardiac adverse events such as HF and MI in the Dana-Farber Cancer Institute, an early Phase II clinical trial. In contrast, the study by Demetri *et al*. (2006) reported no systematic mean decreases on LVEF during a Phase III clinical trial in GIST patients. However, Motzer *et al*. (2007) reported modest declines in LVEF without clinical sequelae in renal cell carcinoma patients. Following this, the study by Chu *et al*. (2007) was able to identify that 11 % of patients, with Imatinib-resistant metastatic GIST, treated with Sunitinib developed cardiac adverse events. Patients were shown to demonstrate a decrease in LVEF and an increase in blood pressure and 47 % of patients developed hypertension (Chu *et al*. 2007). The associated cardiac dysfunction from Sunitinib administration was associated in individuals with histopathology involving myocyte hypertrophy as well as alterations in mitochondrial structure, without inflammatory or fibrotic changes (Chu *et al*. 2007). The study involved a median time to development of all cardiac adverse events at 30.5 weeks, unlike the study by Demetri *et al*. (2006) that contained a median of 10 weeks, suggesting that follow-up studies are required in order to determine if the longer exposure to Sunitinib treatment allowed for patients to develop cardiovascular sequelae (Chu *et al*. 2007).

As highlighted, studies have reported that Sunitinib-treated patients developed LV dysfunction, declines in LVEF, overt HF and the development of symptomatic grade 3-4 HF, however the associated effects by Sunitinib were shown to be reversible after drug discontinuation (Telli *et al*. 2008, Motzer *et al*. 2007). Moreover, the manufacturers of SUTENT (Sunitinib) have reported that Sunitinib demonstrated grade 1 or 2 toxicity in most events of renal cell carcinoma whilst also including reports of grade 3–4 drug-related adverse events (Pfizer 2012, Telli *et al*. 2008). The National Cancer Institute have defined grade I toxicity as any drop in LVEF below normal for LV systolic dysfunction, whilst any reduction below 50 %

represents a grade 2 toxicity for LV systolic dysfunction (CTCAE 2006). Despite reports of LV dysfunction, many studies display few or very little alterations in these haemodynamics, and therefore the underlying mechanisms of cardiotoxicity by Sunitinib are unclear.

As well as cardiac myocytes, mice treated with Sunitinib (40 mg/kg) highlighted the targeting of the pericyte via Sunitinib, inducing microvascular abnormalities and cardiac dysfunction, a result that was not shown during Doxorubicin-induced toxicity (Chintalgattu *et al*. 2013). The authors indicated that the targeting of pericytes by Sunitinib is specific to cardiac muscle, and indicated Sunitinib-induced binding to PDGFR-β. The PDGFR signalling pathway was important in the pro-survival signalling pathway for pericytes (Chintalgattu *et al*. 2013). Inhibition of PDGFR-β signalling was shown to induce similar effects as that shown by Sunitinib, indicating the involvement of the PDGFR-β signalling pathway during Sunitinib treatment (Chintalgattu *et al*. 2013). Moreover, the cytotoxic effect of Sunitinib was inhibited via Thalidomide, as Thalidomide was shown to result in the restoration of pericytes during co-administration, thereby preventing cardiac toxicity and coronary microvascular dysfunction in normal mice and tumour-bearing mice (Chintalgattu *et al*. 2013). However, pericytes are needed to be studied in more detail to understand the precise mechanism impact on coronary microvascular function, as the induced phenotype was reversed following Sunitinib withdrawal (Chintalgattu *et al*. 2013).

Sunitinib's induction of apoptosis via direct anti-VEGFR and PDGFR effects on endothelial cells was demonstrated during the Phase I study by Faivre *et al*. (2006) which demonstrated skin toxicities in patients subjected to doses greater than 75 mg/d, this was hypothesised to result from Sunitinib's ability to induce dermal vessel alteration. Moreover, Sunitinib demonstrated the ability to induce endothelial cell apoptosis in *in-vitro* and in animal tumour models (Rouffiac *et al*. 2004). Furthermore, asymptomatic subungual splinter haemorrhages were observed in patients, this was said to be associated with macroangiopathy (Robert *et al*. 2005, Faivre *et al*. 2006). The resulting thrombosis observed in some patients was associated to result from the increased susceptibility of endothelial cells to damage, this was demonstrated during exposure to the TKI SU5416 (Semaxinib), a similar VEGF inhibitor, in combination with Cisplatin and Gemcitabine (Kuenen *et al*. 2003, Faivre *et al*. 2006). This reinforces the hypothesis that VEGF has a role in

endothelial cell protection (Faivre *et al*. 2006). PDGFRs are expressed in cardiac myocytes, whilst overexpression can signal cardiac myocyte survival (Hsieh *et al*. 2006). Exogenous PDGFR agonists were shown to have no pulmonary toxicity effect (Hsieh *et al*. 2006), therefore indicating the apparent Sunitinib-induced cardiotoxicity may be as a result of an off-target effect, it was highlighted that the RSK family and AMPK could be potential candidates towards the off-target effects of cardiotoxicity (Hsieh *et al*. 2006, Force *et al*. 2007). It can be hypothesised that the inhibition of AMPK results from the inhibition of the pro-survival signals within the heart. The inhibition of the RSK, which acts to signal survival via the inhibition of phosphorylating proapoptotic factor BAD, is said to be responsible for Sunitinib-induced cardiotoxicity (Hasinoff *et al*. 2008, Force *et al*. 2007, Dyck and Lopaschuk 2006, Terai *et al*. 2005). RSK is described to act to signal survival via inhibiting phosphorylation of the pro-apoptotic factor BAD, whilst AMPK is involved in the transducing of pro-survival signalling within the heart (Dyck and Lopaschuk 2006, Terai *et al*. 2005, Hsieh *et al*. 2006, Force *et al*. 2007). The inhibition of RSK and AMPK by Sunitinib could be potentially seen in studies involving relevant concentrations of 25 mg/kg/day and *in-vitro* relevant concentrations of 8–10 μM (Kerkela *et al*. 2009, Fabian *et al*. 2005, Cohen *et al*. 2011).

Additional concerns exist regarding the inhibition of VEGFR signalling in the heart. It was highlighted that during poorly controlled hypertension in patients receiving Sunitinib, studies demonstrated that VEGF-VEGFR signalling was disrupted during the imposition of a pressure load on the heart, moreover capillary density was shown to be reduced (Shiojima *et al*. 2005, Izumiya *et al*. 2006). This was associated with contractile dysfunction, fibrosis and HF (Shiojima *et al*. 2005, Izumiya *et al*. 2006). This therefore indicated that an angiogenic response is necessary when maintaining a normal response of cardiac myocytes to pressure overload, moreover when this does not occur the heart transitions rapidly from compensatory hypertrophy to HF (Shiojima *et al*. 2005, Izumiya *et al*. 2006). Furthermore, the inhibition of the angiogenic response to pressure overload was demonstrated to be mediated via the upregulation of the tumour suppressor p53, therefore suggesting p53 activity within the heart to be important in hypertensive patients (Sano *et al*. 2007, Ventura *et al*. 2007). The study by Sano *et al*. (2007) demonstrated that p53 accumulation is required for the transition from cardiac

hypertrophy to HF, the inhibition of p53 was said to demonstrate beneficial functions following MI.

As highlighted, therapy targeting apoptotic pathways that are regulated by TKs can cause cardiac dysfunction; this includes the non-receptor TK JAK2 and KIT. In leukaemia cell mutations within JAK2 activates members of the STAT3 transcription factors: STAT3 and STAT5 (Lin *et al*. 2000). However, the JAK-STAT signalling pathway is believed to be cardioprotective in the heart, highlighted by the inhibiting of JAK2 in reducing apoptosis in rat models of MI (Negoro *et al*. 2000). Mice engineered to lack STAT3 demonstrated increased susceptibility to cardiac injury via MI and were more susceptible to Doxorubicin-induced cardiotoxicity, however overexpression of STAT3 protected against Doxorubicin-induced cardiotoxicity (Hilifiker-Kleiner *et al*. 2005, Hilifiker-Kleiner *et al*. 2004, Kunisada *et al*. 2000). Following this, STAT3 is said to be vital in maintaining cardiac capillary density via mechanisms such as direct regulation of VEGF expression, the induction of pro-angiogenic cytokines and the suppression of anti-angiogenic activity within the heart (Force *et al*. 2007). However possible cardiotoxicity of JAK2 inhibitors has been flagged, noting the potential of these inhibitors to have activity against several JAK family kinases (Force *et al*. 2007). In clinical development, the most advanced JAK2 inhibitor Lestaurtinib (CEP-701) demonstrated no significant cardiotoxicity in Phase I–II clinical trials in patients with acute myeloid leukaemia and activating FLT-3 mutations (Smith *et al*. 2004).

Sunitinib is known to inhibit the stem cell receptor KIT, expressed on hemangioblasts for haematopoietic stem cells and endothelial progenitor cells (EPCs) (Liang *et al*. 2013). Correct functioning of the KIT receptor is necessary for the mobilisation of EPCs towards sites of injury such as MI (Liang *et al*. 2013). Using a mouse model of a heterozygote allele deletion ($KIT^{W/W\text{-}v}$) and reduced kinase activity subjected to MI, demonstrated impaired post-MI repair and survival (Liang *et al*. 2013). This was attributed to a failure to recruit pro-angiogenic bone marrow-derived stem/progenitor cells (Ayach *et al*. 2006, Fazel *et al*. 2006). Following this, concerns were mentioned regarding KIT inhibition, this was attributed to pathological remodelling of the heart following MI and prevent repair, the inhibition of KIT was said to prevent beneficial homing to areas of injury within

the heart, potentially relevant to patients treated with KIT inhibitors (Force *et al*. 2007).

In summary, Sunitinib was shown to result in abnormalities within the heart including the development of LV dysfunction, declines in LVEF, overt HF and the development of symptomatic grade 3-4 HF. Sunitinib further demonstrated grade 1 or 2 toxicity and was reported to result in grade 3–4 adverse events. The resulting effects are associated with Sunitinib's pro-apoptotic properties as well as Sunitinib's ability to inhibit VEGFR, PDGFR and KIT signalling, as well as mentioned RSK, AMPK and JAK STAT signalling pathway involvement of Sunitinib. For this study, our focus was on the role of AMPK signalling.

The study by Inoki *et al*. (2003) demonstrated AMPK to phosphorylate tuberous sclerosis complex (TSC)-2, thereby increasing the ability of the TSC complex to inhibit the mTOR pathway and inhibiting cell growth to protect against apoptosis during glucose starvation. Growth factors and amino acids activate the mTOR pathway, stimulating translation and cell growth via the activation of ribosomal protein S6 kinase (S6K1) and stimulates the initiation of translation by increasing phosphorylation of elongation factor-4E binding protein 1 (4E-BP1) (Carrera 2004). The serine/threonine protein kinase mTOR is known to regulate cell growth, proliferation and angiogenesis, and is located downstream of AMPK and Akt/PKB, however inhibition of mTOR leads to a feedback activation of Akt/PKB and thereby regulates apoptosis and proliferation as well as cell growth (Larkin *et al*. 2009).

Following this, the inhibition of mTOR has been a common route of renal cell carcinoma treatment however significant toxicity has been reported following inhibition of VEGF and mTOR pathways, particularly with Sunitinib in combination with Temsirolimus, an inhibitor of the mTOR signalling used for treatment of renal cell carcinoma (Patel *et al*. 2009). During the Phase I trial by Patel *et al*. (2009) with Sunitinib at 25 mg (oral, daily) and Temsirolimus 15 mg (Intravenous, weekly) combination resulted in 2 out of 3 patients suffering from severe acneiform erythematous rash and gout/cellulitis requiring hospitalisation. Both doses were lower than the required administration doses of Sunitinib 50 mg and Temsirolimus 25 mg (Pfizer 2014, Torisel 2007). The authors described that these effects may herald from the synergistic effect of the drugs used, as concomitant treatment could

result in additive or synergistic effects on overlapping toxicities, potentially inhibiting compensatory mechanisms that interfere with the development of drug-induced toxicity that is not seen clinically with single agents (Patel *et al*. 2009).

Further studies exist demonstrating the potential for mTOR and TKI inhibition as conjunctive therapy to enhance the antitumor effects. The study by Park *et al*. (2014) investigated the synergistic interaction between the PI3K and mTOR dual inhibitor NVP-BEZ235 and Sunitinib in castration-resistant prostate cancer cells with Docetaxel resistance. The author concluded that the combination of NVP-BEZ235 and Sunitinib resulted in a significant synergistic antitumor effect in the dose-ranges, whilst the half-maximal inhibitory concentrations of both treatments were reduced 7.8 and 6.6-fold (Park *et al*. 2014). It was stated that the combination therapy caused an induction of caspase-dependent apoptosis in Docetaxel-resistant cells; however, Sunitinib did not produce an additional effect on the NVP-BEZ235-mediated inhibition of PI3K/Akt/mTOR phosphorylation (Park *et al*. 2014).

As highlighted, there is a growing concern regarding drug-induced cardiotoxicity and the potential use of adjunctive therapy with Sunitinib treatment. Current studies such as the ASSURE (adjunctive Sorafenib or Sunitinib for unfavourable renal cell carcinoma) study attempted a three-arm randomised trial comprising 1 year of adjunctive therapy with Sorafenib vs. Sunitinib vs. placebo within patients (Porta and Chiellino 2016). The ASSURE study failed to demonstrate a significant difference in disease-free survival in the three treatment arms, whilst the author's indicated a rationale against the use of Sunitinib and suggested kidney cancer may be independent of angiogenesis (Porta and Chiellino 2016). Furthermore, the STRAC trial aimed to compare the disease-free survival time and safety of Sunitinib with placebo for 1 year as a form of adjunctive therapy for patients at a high risk of recurrent kidney cancer following surgery (Porta and Chiellino 2016). The STRAC study demonstrated positive results, of which there was an increase disease-free survival (Porta and Chiellino 2016). Therefore, TKIs are an area of interest, particularly humanised monoclonal antibodies and small-molecule inhibitors against TK receptors, as summarised in Table 1 (Krause e*t al*. 2005).

Table 1 Summary of existing TKIs and associated toxicity

Agent	Class	Target	Cancer Treatment	Associated Toxicity
Sunitinib (Sutent)	TKI	VEGFR1-3, KIT, PDGFRα/β, RET, CSF1R, FLT3.	Renal cell carcinoma, gastrointestinal stromal tumour, pancreatic.	Skin rash, hypertension, haemorrhage and acute coronary syndromes.
Trastuzumab (Herceptin)	Monoclonal antibody	ERBB2.	ERBB2 breast cancer	Neutropaenia and infusion reactions.
Imatinib (Gleevec)	TKI	ABL1/2, PDGFRα/β, KIT.	Chronic myeloid, myelomonocytic and eosinophilic leukaemia and gastrointestinal stromal tumour.	Oedema, nausea, myelosuppression and possible immunosuppression.
Dasatinib (Sprycel)	TKI	ABL1/2, PDGFRα/β, KIT, Src family.	Chronic myeloid leukaemia.	Myelosuppression, oedema, pleural/pericardial effusion, panniculitis, QT prolongation and bleeding.
Nilotinib (Tasigna)	TKI	ABL1/2, PDGFRα/β, KIT.	Chronic myeloid leukaemia.	Myelosuppression, hyperbilirubinemia, rash and QT prolongation.
Sorafenib (Nexavar)	TKI	VEGFR1-3, KIT, PDGFRα/β, FLT3, RAF1, BRAF.	Renal cell carcinoma and melanoma.	Skin rash, hypertension, adrenal dysfunction and hypothyroidism.
Bevacizumab (Avastin)	Monoclonal antibody	VEGFA	Non-small-cell-lung and colorectal.	Haemorrhage, gastrointestinal perforation, poor wound healing, hypertension, neutropaenia and arterial thromboembolism.
Lapatinib (Tykerb)	TKI	EGFR, ERBB2	Breast cancer.	Skin rash and diarrhoea.

Gefitinib (Iressa)	TKI	EGFR	Non-small-cell-lung cancer.	Skin rash, diarrhoea, nausea and interstitial lung disease.
Erlotinib (Tarceva)	TKI	EGFR	Non-small-cell-lung and pancreatic cancer.	Skin rash, diarrhoea, nausea and interstitial lung disease.
Cetuximab (Erbitux)	Monoclonal antibody	EGFR	Colorectal cancer, squamous cell carcinoma of the head/neck.	Skin rash, infusion reactions, interstitial lung disease and hypomagnesaemia.
Panitumumab	Monoclonal antibody	EGFR	Colorectal cancer.	Skin rash.

Table 1: Summary of existing TKIs and TK-targeting agents which have been associated with cardiotoxicity; QT prolongation, prolongation of the QT interval on electrocardiogram that may predispose to arrhythmia.

1.9. Metformin therapy and Metformin-induced cardioprotection

With the vast amount of TKIs and similar agents being associated with cardiovascular events, it is important to prevent these associated symptoms in patients, one approach is to develop an adjunctive or synergistic approach to using TKIs, particularly with other non-competitive agents. A large epidemiological trial involving 4075 patients with T2DM with Metformin treatment improved cardiovascular prognosis in comparison to glucose-lowering agents Chlorpropamide, Glibenclamide or insulin and was able to reduce the incidence of MI within patients by 39 % (UK Prospective Diabetes Study 1998). Several studies have highlighted the cardioprotective properties of Metformin treatment, particularly in ischaemia-reperfusion studies (Paiva *et al*. 2009, Paiva *et al*. 2010, Bhamra *et al*. 2008, Barreto-Torres *et al*. 2012).

The biguanide Metformin hydrochloride, is currently prescribed for the treatment of insulin resistance, T2DM and impaired glucose intolerance at oral dosages of 500 mg twice a day or 850 mg once a day (Figure 7) (Giannarelli *et al.* 2003, FDA nd). Metformin is prescribed to over 100 million patients worldwide and has been in clinical use for over 50 years (Rena *et al.* 2013) and has been in use since 1957 in Europe and since 1995 in the USA (Pernicova and Korbonits 2014). Metformin decreases hepatic glucose production and intestinal absorption of glucose, whilst improving insulin sensitivity by increasing peripheral glucose uptake and utilisation (FDA nd). 500 mg oral dosage of metformin, under fasting conditions, has an absolute bioavailability of 50–60 %, whilst food has been described to decrease the extent of and slightly delay absorption, approximately a 40 % lower mean peak concentration (C_{MAX}), 25 % lower area under the plasma concentration versus time curve, 35 minute prolongation of time to peak plasma concentration (T_{MAX}) following administration of a single 850 mg tablet with food compared to the administration during fasting (FDA nd).

Metformin's mode of action has been described to reduce insulin levels and promote improved receptor activity, both direct and indirect effects via insulin action and steroidogenic enzymatic activity and improve nitric oxide (NO) vasodilator effects in the endothelium (Giannarelli *et al.* 2003, Traub 2011). It has been hypothesised that Metformin principally acts on the liver, inhibiting hepatic gluconeogenesis, as well

as binding to ACC activity and suppressing fatty acid production (Giannarelli *et al.* 2003), whilst optimising hepatic glucose metabolism by enhancing the AMPK signalling pathway, suppressing hepatic gluconeogenesis (Inzucchi *et al*. 1998). Furthermore, Metformin acts upon skeletal muscle and adipose tissue to inhibit lipid production and stimulate glucose transport and uptake (Yuan *et al.* 2003, Guigas *et al.* 2004). Metformin has been suggested to reduce lipid uptake or synthesis in the intestine and in the hepatocytes, during therapy the observed improvement of obesity, abdominal obesity, with a subsequent decreased release of free fatty acids from adipose tissue could help to explain the improvement of lipid profile during Metformin treatment (Shaker *et al.* 2010). Serum concentrations of insulin HOMA and HOMA-beta-cell % were decreased significantly after three months of treatment (Shaker *et al.* 2010). It was suggested that the reduction in hyperinsulinemia observed may be due to improvements in hepatic extraction and insulin sensitivity during therapy with Metformin (Shaker *et al.* 2010). It was also observed that Metformin reduced basal levels of free fatty acids, which have a role in reducing glucose disposal in skeletal muscle, leading to impaired insulin sensitivity (Shaker *et al.* 2010). Furthermore, a study by Ferreira *et al*. (2013) was able to highlight an increase in PI3K and GLUT4 expression among cells cultured with Metformin for 24 and 48 hours. Following this, reports have further indicated Metformin to be able to activate Rab4, involved in translocation of intracellular GLUT4 to the cell membrane (Lee *et al*. 2011).

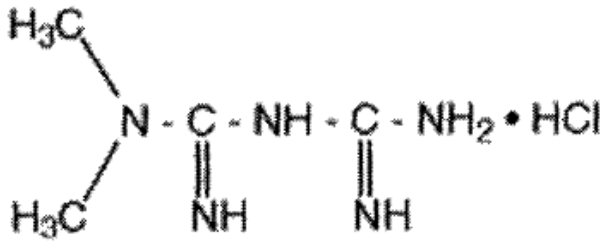

Figure 7: Metformin Hydrochloride *N*-dimethylimidodicarbonimidic diamide hydrochloride, $C_{14}H_{11}N_5HCl$, molecular weight 165.63, pH of a 1 % aqueous solution of metformin hydrochloride is 6.68, pK_a 12.4, (FDA nd).

Metformin's properties are believed to stem from the involvement on cellular ATP levels (Rena *et al*. 2013), it was deduced that Metformin was able to suppress hepatic glucose output, with the inhibition of complex I within the mitochondrial electron transport chain (Figure 9) (Owen *et al*. 2000, El-Mir *et al*. 2000). Further to this, when compared to the complex II substrate Succinate, that can bypass complex I inhibition, Metformin was shown to inhibit mitochondrial oxidation of glutamate more effectively (Owen *et al*. 2000, El-Mir *et al*. 2000). The magnitude of inhibition of gluconeogenesis is believed to be correlated to the extent of inhibition of the respiratory chain, suggesting that the change in energy results in the insufficient flux of ATP for gluconeogenesis (Owen *et al*. 2000). The inhibition of gluconeogenesis and mitochondrial inhibition may be contributed by ATP-, ADP- and AMP-independent effects on pyruvate carboxylase (Owen *et al*, 2000). Mitochondrial inhibition is further known to result in the accumulation of lactate from glycolysis, contributing to respiratory chain inhibition (Owen *et al*, 2000). Mitochondrial oxidation of glutamate and malate inhibition by Metformin, was suggested to result from a time-dependent and concentration-dependent inhibition of the respiratory chain to restrain hepatic gluconeogenesis and increase glucose utilisation in peripheral tissue suggesting Metformin to be less toxic than other complex I inhibitors and the reduced rate of lactic acidosis (Owen *et al*, 2000).

Metformin was shown to reduce the risk of MI in overweight patients with T2DM in comparison to diet therapy in overweight diabetic patients (UKPDS 1998). Moreover, Metformin consists of vasoprotective as well as cardioprotective properties following the results of the United Kingdom Prospective Diabetes study which demonstrated Metformin to reduce all-cause mortality by 36 %, mortality associated with type-2 diabetes by 42 %, myocardial infarction by 39 % and stroke by 41 % respectively (UKPDS 1998). Metformin was also shown to reduce all-cause mortality and CV mortality in T2DM patients in comparison to Sulphonylurea therapy (Johnson *et al*. 2002) and has been described to show beneficial changes in blood rheology, serum lipid profile and anti-ischaemic effects (Anfossi *et al*. 2010). However, in contrast to this, two meta-analyses have failed to determine the benefit of Metformin therapy on all-cause or cardiovascular-related mortality, or on diabetes-relates macrovascular complications, contributing towards the debate

regarding Metformin's ability to reduce the occurrence of cardiovascular disorders (Boussageon *et al*. 2012, Selvin *et al*. 2008).

Metformin was shown to modulate inflammation by suppressing interleukin (IL)-1β-induced IL-8 production, cyclic AMP (cAMP)-induced mRNA expression and aromatase activity, limiting the development of endometriosis (Takemura *et al*. 2007). Metformin was stated to exert an anti-inflammatory role by reducing pro-inflammatory cytokine secretion, and potentially inhibiting endometriosis by suppressing both ovarian and local production of oestrogen as exhibited by Metformin's ability to suppress cAMP-stimulated aromatase activity in endometriotic stromal cells (Takamura *et al*. 2007). Metformin treatment improved lipid profiles, vascular-smooth-muscle, hypercoagulation, and platelet function (Kirpichnikov *et al*. 2002), whilst improving relaxation and calcium handling within rat cardiac myocytes during hyperglycaemia (Ren *et al*. 1999) and reduce the generation of ROS (An *et al*. 2006, Kukidome *et al*. 2006, Rosen and Wiernsperger 2006).

The mechanism of cardioprotection by Metformin has only recently been investigated. It was reported that Metformin inhibits the opening of the mPTP during reperfusion by activating the protein kinase Akt (Bhamra *et al*. 2008), whilst protecting cells against calcium and oxidative stress (Detaille *et al*. 2005, Guigas *et al*. 2004). Therefore, efforts have been concentrated towards preventing the opening of mPTP channel, with studies highlighting the resulting effect to reduce infarction size (Hausenloy *et al*. 2002, Hausenloy *et al*. 2004). Metformin was shown to be dependent on the activation of AMPK; the phosphorylation of AMPK was shown to be mediated by the increase in the cytosolic AMP concentration (Zhang *et al*. 2007). Metformin was further shown to promote interaction between the upstream activating kinase LKB1 and AMPK, for AMPK activation (Zou *et al*. 2004). The stimulation of the adenosine receptor was shown to be an important mechanism in infarct size-limiting effects of ischaemic pre- and post-conditioning (Yellon and Downey 2003), whilst adenosine receptor stimulation was shown to activate Akt (Yang *et al*. 2004) and inhibit the opening of mPTP (Hausenloy *et al*. 2002). The survival protein kinase Akt is known to contribute towards the reperfusion injury salvage kinase pathway, reducing MI size (Hausenloy and Yellon 2004).

A study involving Metformin treatment in T2DM and normal-glycaemic rat hearts was able to indicate the reduction in MI infarct size, when administered at the time of reperfusion (Bhamra *et al*. 2008). Metformin-treated ischaemia-reperfusion hearts were shown to improve respiration and respiratory control index, an effect that ischaemia-reperfusion depletes as a result of decreasing efficiency of respiratory coupling and ATP synthesis (Barreto-Torres *et al*. 2012). Furthermore, Metformin demonstrated cardioprotective effects during treatment in perfused rat hearts and rat cardiac myocytes via the phosphorylation of Akt (Bhamra *et* al. 2008). Studies have shown that Metformin is able to exert cardioprotection in rat models following ischaemia-reperfusion via activation of PPARα (Barreto-Torres *et al*. 2012, Oidor-Chan *et al*. 2016). PPARα is highly expressed in the heart as well as other organs, and PPARα ligands were shown to reduce myocardial ischaemia-reperfusion injury in mice models (Yue *et al*. 2003). PPARα was suggested to prevent mPTP formation via directly interacting with mPTP components, voltage–dependent anion channel (VDAC), adenine nucleotide translocase (ANT) and/or cyclophilin D (CyPD) and could also indirectly by activating signalling pathways PI3K/Akt and NO production (Barreto-Torres *et al*. 2012, Bulhak *et al*. 2009). Metformin was further able to decrease cardiac myocyte apoptosis and increase NO (Gundewar *et al*. 2009), improving endothelial function whilst maintaining myocardial energy production during ischaemia (Hardie 2003). In addition to this, clinical studies demonstrated Metformin's ability to reduce plasma dipeptidyl peptide-4 activity and increase circulating levels of GLP-1, associated with cardioprotective effects (Maida *et al*. 2011). Metformin was shown to decrease myocardial damage in cultured myocytes, via inhibiting apoptosis (Sasaki *et al*. 2009), whilst LV fractional shortening and LV end-diastolic pressure were improved in dogs treated with Metformin, however another study demonstrated cleaved caspase-3 levels were only slightly reduced with Metformin treatment (Yin *et al*. 2011).

1.10. The anticancer properties of Metformin.

As well as the discussed cardioprotective properties, Metformin has anti-cancer properties. Metformin-treatment is associated with a decrease in incidences of cancer and cancer-related mortality in diabetic patients (Bowker *et al*. 2006, Evans *et al*. 2005, Janjetovic *et al*. 2011). Since 2005 Metformin has been associated with a reduced incidence of cancer, following this, over 173 clinical trials involving Metformin and cancer have been reported (U.S. National Library of Medicine nd). Metformin was reported to reduce this risk of cancer development and cancer–related mortality when compared to other diabetes treatment such as sulfonylureas (Evans *et al*. 2005, Bowker *et al*. 2006, Monami *et al*. 2009, Wright and Stanford 2009, Giovannucci *et al*. 2010). Metformin's ability to act on the liver to lower systemic glucose levels and improve secondary hyperinsulinemia has been associated with preventing tumour growth and progression, hyperinsulinemia itself is associated with an adverse prognosis in breast, colon and prostate cancer (Pollak 2012). This results in Metformin being able to affect insulin-sensitive neoplastic tissue without accumulating cancer cells (Pollak 2012). Metformin was further said to demonstrate cell proliferating inhibition properties, reducing colony formation and result in partial cell cycle arrest in cancer cell lines (Zakikhani *et al*. 2006, Alimova *et al*. 2009, Liu *et al*. 2009). In contrast, the population-based study involving diabetic patients with breast cancer, aged 65 years and older, failed to demonstrate an association between improved survival and Metformin use (Lega *et al*. 2013). Metformin's ability to attenuate tumour genesis and chemoprotective properties are poorly understood, especially as concentrations used in *in-vitro* experiments are not able to be translated into patients (Pollak 2012). Further limitations include the varying degree of tissue expression of transporters mediating Metformin uptake, in normal and tumour cells, which can be influenced by drugs including antibiotics (Nies *et al*. 2012). The poor uptake into target cells may limit the therapeutic ability of Metformin in cancer (Nies *et al*. 2012).

The population study by Bowker *et al*. (2006) demonstrated that cancer-related mortality was shown to be 3.5 % (245 out of 6,969) for patients treated with Metformin compared to patients not receiving Metformin, and suggested that Metformin has the ability to decrease the incidence of cancer and cancer-related

mortality in diabetic patients (Bowker *et al*. 2006, Evans *et al*. 2005, Janjetovic *et al*. 2011). Moreover, the study by Keizman *et al*. (2016) demonstrated an increase in overall survival for renal-cell carcinoma patients receiving Metformin compared to patients not receiving Metformin. Metformin is suggested to have anti-neoplastic properties by affecting cancer cells by either decreasing insulin levels or inhibiting cancer cell proliferation and apoptosis (Margel *et al*. 2013), via the activation of AMPK to inactivate enzymes associated with ATP consumption and the inhibition of mTOR/mTOR complex 1 (mTORC1) pathway in tumourigenesis (Zakikhani *et al*. 2006, Vazquez-Martin *et al*. 2009). From this, it can be suggested that AMPK activation may be having a potential anti-cancer and cardioprotective properties.

The activation of AMPK is known to inhibit mTOR activity; mTOR is regulated by cellular energy levels, nutrients, oxygen and growth factors (Hay and Sonenberg 2004). The dysregulation of mTOR, and mTOR-associated signalling pathways, is said to result in uncontrolled cell growth and proliferation (lagan and Manning 2016). PI3K3CA mutations or loss of expression of the tumour suppressor phosphatase and tensin homolog (PTEN) can lead to uncontrolled mTOR activity in breast cancer (Neshat *et al*. 2001, Bjonsti and Houghton 2004). Following this, inhibiting pathways of AMPK activation and mTOR inhibition has been an area of interest to prevent and inhibit cancer cell growth. Studies have demonstrated Metformin's ability to inhibit growth of breast cancer cells in *in-vitro* and tumours *in-vivo* (Buzzai *et al*. 2007, Dowling *et al*. 2007, Zakikhani *et al*. 2006). Patients receiving neoadjuvant chemotherapy for breast cancer demonstrated that diabetic patients with breast-cancer and receiving Metformin during treatment had a greater pathological complete response rate than diabetic patients not receiving Metformin (Jiralerspong *et al*. 2009). Patients receiving Metformin demonstrated a pathological response rate of 24 % compared to 8 % for patients not receiving Metformin (Jiralerspong *et al*. 2009). It was proposed that the activation of AMPK leads to the suppression of metabolic processes requiring high amounts of ATP as well as the exerting inhibitory effects on the mTOR pathway; phosphorylating TSC2 and Raptor (Gonzalez-Angulo and Meric-Berstam 2010).

In-vivo experiments involving orally administered or intraperitoneal Metformin in mice demonstrated a tumour growth inhibition of up to 55 % (Ben Sahra *et al*. 2010, Gonzalez-Angulo and Meric-Berstam 2010). Metformin was further shown to inhibit

growth of MCF-7 human breast cancer cell lines, administration of growth inhibitory concentration of Metformin was suggested to decrease protein synthesis, blocking growth and proliferation via AMPK activation and mTOR inhibition (Zakikhani *et al*. 2006, Dowling *et al*. 2007). Moreover, Metformin was shown to enhance the antiproliferative ability of Doxorubicin during co-administration in non-stem and cancer stem cell lines, whilst also reducing tumour mass and prolonged remission in a xenograft mouse model, in comparison to Doxorubicin administration alone (Hirsch *et al*. 2009).

A summary of Metformin's anticancer ability is presented in Figure 8. The proposed mechanism of action of Metformin is believed to stem from Metformin-induced activation of the AMPK pathway in tumour cell lines, resulting in growth inhibition via potentially inhibiting protein synthesis (Dowling *et al*. 2007, Zakikhani *et al*. 2006, Alimova *et al*. 2009, Liu *et al*. 2009). Metformin's other suggested anti-neoplastic properties may result from Metformin's ability to either decrease insulin levels or inhibiting cancer cell proliferation and apoptosis, via the activation of AMPK to inactivate enzymes associated with ATP consumption and the inhibition of mTORC1 pathway in tumourigenesis (Zakikhani *et al*. 2006, Vazquez-Martin *et al*. 2009). Although the mechanisms outlining the direct role of AMPK in anticancer treatment is not fully established, studies have suggested Metformin to have a direct action on cancer cells, involving AMPK-dependent and AMPK-independent mechanisms, whilst AMPK is suggested to interfere with cancer cell growth by activating HIF-1α, tumour protein p53 and DICER1 (Pierotti *et al*. 2013, Bar-Peled *et al*. 2012, Pernicova and Korbonits 2014). AMPK has further been suggested to act as a mediator towards the nuclear factor–κB (NFκB)/IL-6 pathway, delaying the malignant transformation via attenuating the inflammatory feedback loop (Pernicova and Korbonits 2014).

As described, the mTOR pathway is able to be suppressed via an AMPK-dependent manner, a potential anti-neoplastic effect of Metformin (Wang *et al*. 2011). Metformin is shown to reduce types of neoplasm with the reductions being associated with reductions in the level of mTORC1 (Rogalska *et al*. 2016). The mTOR pathway is known to contribute towards the progression and proliferation of tumours (Chiang and Abraham 2007). In reference to cancer, the mTOR inhibition is described to interfere with protein synthesis and tumour cell proliferation, with loss

of LKB1 shown to be frequent in cancer (Wang *et al*. 2011). As mTOR is the catalytic subunit of mTORC1 and mTORC2, these complexes are important in regulating cellular growth, whilst integrating the input from hormonal signalling and energy-sensing pathways, such as insulin–like growth factor (IGF)-1-2 and AMPK (Inoki *et al*. 2002). The activation of TSC1 and TSC2 creates an mTOR-inhibiting complex, thereby down-regulating mTORC1, in an AMPK-dependent manner (Gwinn *et al*. 2008).

As touched upon, IGF-1 insulin signalling is inhibited by Metformin via AMPK-dependent phosphorylation of insulin receptor substrate (IRS)-1, which is known to transmit signals from insulin and IGF-1 receptor to the P13K-Akt pathway, potentially down regulating mTOR signalling (Ning and Clemmons 2010). In contrast to this cascade, reports have highlighted multiple regulatory feedback loops that counteract the antitumor effect during Metformin exposure (Vazquez-Martin *et al*. 2009). AMPK targets the tumour suppressor protein p53, which is able to activate genes for the inhibition of Akt and mTORC1 pathways; however, the role of Metformin in p53 activation is not fully established (Liang *et al*. 2007). Metformin is further known to induce the expression DICER1, involved in microRNAs synthesis, loss of function mutation is known to result in the complex tumour syndrome (Blandino *et al*. 2012). As tumours, inflammatory cells and other rapidly dividing cells favour glycolysis to oxidative phosphorylation as an energy source for metabolism, this promotes fatty acid synthesis and intermediates for proliferation, including mTORC1-activated HIF-1α, thereby promoting the expression of GLUT-1 and mono carboxylate transporter 4 (MCT4) for lactic acid transport (Thomas *et al*. 2006). The activation of AMPK was shown to inhibit the proto - oncogenic c-MYC and HIF-1α (Blandino *et al*. 2012). In summary, the proposed mechanism of Metformin results in the inhibition of the PI3K-Akt-mTOR pathway, moreover AMPK phosphorylates TSC2 which inhibits mTORC1, thereby decreasing in protein synthesis and cell growth (Yoshida *et al*. 2011, Patel *et al*. 2015).

Within T2DM patients, variation of the glycaemic response to Metformin was associated with the presence of a common genetic variant of the ataxia telangiectasia mutated (ATM) gene locus (Zhou *et al*. 2011). From this, ATM activation was said to be involved in Metformin-dependent signalling, as ATM encodes the tumour suppressor protein component of the DNA-damage response

for DNA repair and cell-cycle control (Zhou *et al*. 2011). The ATM mutation was indicated to be present in ataxia telangiectasia (Zhou *et al*. 2011). This neurodegenerative condition has been associated with the predisposition to cancer, insulin resistance and T2DM, whilst both AMPK-dependent and -independent mechanisms were suggested to be involved in the ATM-mediated reparatory effect (Zhou *et al*. 2011). For this, the cellular stressor effects of Metformin are involved in activating the repertory process, a protective effect against pre-malignant to malignant transformation (Vazquez-Martin *et al*. 2011). AMPK acts to inhibit the rate-limiting steps during lipogenesis, including sterol regulatory element-binding protein (SREBP)-1 via mTOR in hepatic cells, thereby decreasing lipid deposition (Scot *et al*. 2004). Repressing AMPK is not only known to prevent the described effects but also accelerates fatty liver developments (Scot *et al*. 2004). ATP depletion results in AMPK activation of catabolic pathways to generate ATP and switch-off ATP-consuming mechanisms (Hardie and Hawley 2001). Metformin is known to improve hepatic steatosis via increasing AMPK phosphorylation (Zhou *et al*. 2001), whilst constitutively active AMPK can potentially inhibit the expression of lipid synthesising gene SREBP-1 and attenuating oleic acid-induced lipid deposition in rat hepatocytes (Jung *et al*. 2011).

Targeting the activation of the LKB1-AMPK axis is an area of interest in cancer, in particular Metformin is used to activate AMPK via an LKB1-dependent mechanism (Green e*t al*. 2011). As we mentioned, Metformin inhibits the mitochondrial respiratory chain complex 1, resulting in the generation of ROS which activates PKC, inducing LKB1 phosphorylation, required for LKB1 nucleo-cytoplasmic translocation and AMPK activation (Memmott *et al.* 2009, Dowling *et al*. 2007). The LKB1–AMPK-mTOR signalling pathway links cellular metabolism and energy status in the signal transduction pathway required for cell growth, proliferation and autophagy (Shackelford and Shaw 2009). The LKB1-AMPK axis is shown to be functional in hematopoietic cancers such as myeloid leukaemia (Green *et al*. 2011). LKB1 is a key upstream activator of AMPK in low ATP conditions, upon an increase in AMP/ATP ratio, AMP binding results in an allosteric change of AMPK and exposes AMPK$_{\alpha}$ Thr^{172} residue for phosphorylation via LKB1 and activates AMPK (Green e*t al*. 2011). AMPK acts as a tumour suppressor kinase via the p53-dependent regulation of cell cycle (Tiainen *et al*. 2002, Jones *et al*. 2005).

Metformin was reported to improve cancer responses during radiation therapy, during the investigation of MCF-7 human breast cancer cells in combination with ionising radiation (Song *et al*. 2012). Another study using Metformin in combination with ionising radiation on non-small cell lung cancer cells, demonstrated that Metformin inhibited cell and tumour growth whilst also sensitising cells to ionising radiation (Storozhuk *et al*. 2013). Metformin was also shown to improve pathologic complete response rate in patients undergoing rectal cancer treatment, of which patients taking Metformin demonstrated a 35 % increase in pathological complete response rate, as well as an increase in disease-free and overall survival compared to patients not taking Metformin (Skinner *et al*. 2013).

Metformin's ability was hypothesised to result from the downregulation of the hyperactive PI3K-Akt-mTOR pathway (Song *et al*. 2012). It contrast to studies demonstrating the inability of Metformin in co-treatment studies, Metformin was shown to enhance apoptosis induced by chemotherapy agents Paclitaxel and Cisplatin in endometrial cancer, the authors highlighted the involvement of glyoxalase 1 expression but were unable to conclude the direct mechanism (Dong *et al*. 2012). Metformin was shown to successfully induce cell death amongst cancer stem cells in mouse xenograft cancer models, whilst further prolonging tumour remission in combination with chemotherapy agents, via possible regulation of the inflammatory pathways (Iliopoulos *et al*. 2011, Hirsch *et al*. 2009). Moreover, Metformin is believed to inhibit cancerous transformation via attenuation of the inflammatory feedback loop (Hirsch *et al*. 2013). This signalling cascade is known to be mediated by the transcription factor NFκB with the down-stream cytokine IL-6 (Hirsch *et al*. 2013). However, this inhibitory effect remains more effective in cancer stem cells than non-cancer stem cells, as the degree of sensitivity of the transformed cell lines to Metformin is said to be reliant upon the degree of immune cell-mediated tumour inflammation, reflected by IL-6 levels (Hirsch *et al*. 2013). This suggests Metformin's ability to activate AMPK varies upon whether there is a metabolic difference between cancer stem cells and regular stem cells (Anastasiou 2013). The study by Misirkic *et al*. (2012) demonstrated that an autophagic response is triggered via AMPK-dependent manner in U251 human glioblastoma cell lines, whilst also inhibiting Simvastatin-induced apoptosis, suggests a cytoprotective role of AMPK-activation of autophagic response during statin-mediated apoptosis.

AMPK-induced activation of autophagy was followed by Raptor activation with subsequent decreases in mTOR and S6K phosphorylation (Misirkic *et al*. 2012). Furthermore, the authors concluded that siRNA-mediated downregulation of AMPK prevented inhibition of mTOR and S6K activation via Raptor, however Akt phosphorylation was said to not be affected by AMPK siRNA. The authors concluded that this result excluded the role of Akt inhibition in early AMPK-dependent induction of autophagy.

The *in-vitro* study by Janjetovic *et al*. (2011) demonstrated the use of Metformin in combination with Cisplatin cotreatment, in HL60 human leukaemia cancer cell lines. Janjetovic *et al*. (2011) indicated the role of serine/threonine Akt-activation, inhibiting apoptosis via phosphorylation and inactivation of targets BAD, forkhead transcription factors, c-Raf and caspase-9 (Datta *et al*. 1997, Brunet *et al*. 1999, Zimmermann and Moelling 1999, Cardone *et al*. 1998). The components of the PI3K/Akt pathway are said to be amplified in tumours sensitive to chemotherapy (Yuan and Cantley 2008, Janjetovic *et al*. 2011). With the knowledge that ROS are involved in some chemotherapy-induced cell death, Metformin-mediated activation of Akt was demonstrated to decrease ROS production in Cisplatin-treated cells (Janjetovic *et al*. 2011). Akt has been associated with protecting mPTP opening against oxidative stress (Bhamra *et al*. 2008, Wang *et al*. 2009, Muders *et al*. 2009). Although AMPK is involved in VEGF-induced Akt phosphorylation in breast cancer cells (Levine *et al*. 2007), Metformin was not able to increase Cisplatin-induced AMPK activation, with AMPK activators also failing to mimic Metformin-mediated Akt activation and cardioprotection, Akt-mediated anti-apoptotic action of Metformin was deduced to be independent of AMPK (Janjetovic *et al*. 2011). The finding suggest that Metformin has the ability to reduce ROS-induced cell death and apoptosis, potentially preventing cardiac myocyte death and protecting the myocardium from drug-induced toxicity.

The study by Shi *et al*. (2012) demonstrated co-administration of Metformin with Doxorubicin was able to enhance lymphoma cell response, the authors stated that combined treatment triggered lymphoma cell autophagy. Autophagy is described as the cellular self-digestive process, and can play a role in cell death, whilst the mTOR pathway is said to regulate cell autophagy under stress conditions, including in response to anticancer agents (Shi *et al*. 2012, Hanahan and Weinberg 2011). In

particular inhibition of mTOR is known to result in the activation of autophagic responses (He *et al*. 2009, Meijer and Codogno 2004). In relation to this, Metformin is considered to target tumour cells by amplifying chemotherapy-induced AMPK activation and inducing tumour cell apoptosis, as well as inhibiting tumour development via autophagy (Shi *et al*. 2012, Rocha *et al*. 2011, Tomic *et al*. 2011). Autophagy is important as it plays an alternative route of apoptosis towards cell death, whilst activation is considered to improve chemotherapy-sensitising effects of certain anticancer agents (Shi *et al*. 2012, Lin *et al*. 2010). mTOR is considered to act as a negative regulator of autophagy, therefore inhibition of mTOR by AMPK activation stimulates autophagy, via phosphorylation of downstream targets Raptor as discussed (Shi *et al*. 2012, Viana *et al*. 2008, Shackelford and Shaw 2009).

Figure 8: Summary of Metformin's antiproliferative properties. Metformin is proposed to inhibit the mTOR pathway directly and indirectly. Upon AMPK activation, processes requiring high amounts of ATP are surpassed, inhibiting the transcription of gluconeogenesis genes in the liver as well as increasing glucose uptake in skeletal muscle, reducing circulating levels of glucose and increasing insulin sensitivity and reducing hyperinsulinemia. Within the cancer cell, dysregulated mTOR signalling promotes cell growth and proliferation; TORC1 directly regulates cell growth and contains Raptor and PRAS40 required for the suppression of mTOR activity. AMPK activation, following low levels of ATP, switches off mTOR signalling over positive effects of amino acids or growth factors via phosphorylation of TSC2, stimulating Ras homolog enriched in brain (Rheb)-GAP activity. In the absence of TSC2, AMPK activation occurs through Raptor phosphorylation by Metformin, directly effecting mTOR kinase activity Gonzalez-Angulo and Meric-Berstam 2010).

1.11. AMPK-dependent activation during Metformin–induced cardioprotection.

As highlighted, the AMPK is known to be a critical energy sensor and regulator of energy homeostasis (Zhou *et al*. 2001, Hardie *et al*. 2012). ATP depletion results in AMPK activation of catabolic pathways to generate ATP and switch-off ATP-consuming mechanisms. This results in the increase in cellular ADP: ATP and AMP:ATP ratios, via decreasing the catabolic production of ATP, through nutrient depression and exposure to mitochondrial toxins, or via promoting ATP consumption through muscle contraction (Hardie *et al*. 2012).

Zhou *et al*. (2001) highlighted that Metformin stimulated AMPK activation, whilst the stimulation was associated with the inhibition of glucose production in rat hepatocytes. Metformin was shown to have an indirect effect on AMPK, by increasing the AMP: ATP and ADP:ATP ratios in engineered cell lines expressing AMPK complexes with either wild-type γ2 isoform or Arg531>Gly mutation, thusly expressing γ2 complexes insensitive to the effects of AD and AMP phosphorylation (Hawley *et al*. 2010). Furthermore, AMPK activation was reduced with liver-specific *LKB1* knockout hyperglycaemic mice, with Metformin failing to produce glucose-lowering effects in these mice models due to the absence of LKB1 (Shaw *et al*. 2005). It was proposed that LKB1-AMPK signalling regulates the expression of gluconeogenic genes via the regulation of transcription coactivator cAMP response element-binding protein-regulated transcription coactivator 2 (CRTC2) (Shaw *et al*. 2005, Altarejos and Montminy 2011). CRTC2 is in a dephosphorylated state, localised in the nucleus, and enhances gluconeogenic genes such as PPARγ coactivator-1α (Rena *et al*. 2013). Insulin intake is known to switch-off glucose production via inhibiting gluconeogenic gene programmes, CRTC2 and forkhead box-O1 (FOCO1) proteins (Lin *et al*. 2011). Metformin is known to switch-off gluconeogenesis, independent of insulin/Akt signalling via the LKB1-AMPK pathway (Shaw *et al*. 2005).

Metformin administration in AMPKα1/α2 *Prkaa1/2* or LKBI knock-out mice demonstrated a glucose-lowering effect, compared to controls; highlighting Metformin's effect on gluconeogenesis in the liver is mainly mediated through AMPK-dependent kinases (Foretz *et al*. 2010). Furthermore, hepatocytes lacking AMPK were treated with Metformin and displayed inhibition of glucose production, with hepatocytes lacking LKB1 were found to have higher rates of basal-unstimulated and cAMP-stimulated glucose production (Foretz *et al*. 2010). However, when considering knock-out mice studies, inactivation of critical metabolic genes could result in compensatory adaptations in alternate pathways in maintaining glucose homeostasis (Rena *et al*. 2013), indicating the importance of establishing the role of AMPK in Metformin's cardioprotective properties.

Figure 9: The anti-hyperglycaemic effect of Metformin and activation of AMPK on the liver cell (Modified figure from Rena *et al*. 2013). Metformin is transported into hepatocytes via organic cation transporter-1 (OCT1) inhibiting the mitochondrial respiratory chain complex 1 formation. The deficit in energy production is known to be balanced by a reduction in energy consumption by the cell, including gluconeogenesis within the liver. This results in the decrease in ATP and increase in AMP concentration, further contributing to the inhibition of gluconeogenesis due to the ATP deficit. The increase in AMP concentration acts as a signalling mediator to inhibit cAMP-PKA signalling via suppressing adenylate cyclise. The increase in AMP concentration further acts to inhibit the gluconeogenic enzyme fructose-1, 6-biphosphatase (FBPase), whilst also activating AMPK, all leading to the inhibition of gluconeogenesis and lipid cholesterol synthases (Rena *et al*. 2013).

Metformin stimulates the insulin-induced component of glucose uptake into both skeletal muscle and adipocytes (Klip and Leiter 1990). This demonstrated significant increase in diabetic patients, indicating that the drug has an enhanced effect in a hyperglycaemic state (Klip and Leiter 1990). Metformin further increases the insulin-dependent portion of glucose oxidation, whilst elevating the uptake of non-metabolisable analogues of glucose and increasing glucose-analogue transport independently of insulin (Klip and Leiter 1990). The antihyperglycaemic effects shown by Metformin are observed in many but not all guanidine-containing compounds, however guanidine itself was shown to be toxic for clinical use (Watanabe 1918), as well as diguanides such as Synthalin A and B (Bischoff *et al*. 1928), before attention was shifted to Metformin Rena *et al*. 2013). The inhibition of the mitochondrial complex I is known to reduce NADH oxidation, thereby lowering the proton gradient through the inner mitochondrial membrane and the proton-driven ATP synthesis, this shifts the ATP: ADP: AMP equilibrium towards the increased AMP synthesis via adenylate kinase, reducing the energy charge of the cell (Owen *et al*. 2000). The raised AMP levels are known to inhibit adenylate cyclise, preventing the converting of ATP to cAMP, thereby ultimately reducing cAMP levels, as shown in Figure 9 (Miller *et al*. 2013). The resulting effect reduces levels of PKA activity and inhibits glucagon-dependent glucose output from hepatocytes, ultimately inhibiting gluconeogenesis (Miller *et al*. 2013). The stimulation of PKA phosphorylates protein targets to increase hepatic glucose output (Miller *et al*. 2013).

AMPK is known to be activated by pathological intracellular stress that depletes cellular ATP, including metabolic poisoning, oxidative stress, hypoxia and nutrient deprivation (Hardie 2004). AMPK is also known to inhibit energy consuming pathways, particularly within the heart this can be an issue due to the high demand in ATP, however AMPK activation can lead to an increase in glucose and fatty acid metabolism during this demand in ATP (Dyck and Lopaschuk 2006). Little energy reserves are present within the heart, therefore if ATP production were ceased the ATP supply within the heart is said to be exhausted (Dyck and Lopaschuk 2006). With this, cardiac AMPK is activated during ischaemia, the activation leads to the stimulation of glucose uptake, glycolysis and fatty acid oxidation (Dyck and Lopaschuk 2006). However, during ischaemia these metabolic effects can be

harmful as ischaemia is known to induce apoptosis in the heart, whilst AMPK is suggested to result in either pro- and anti-apoptotic effects (Capano and Crompton, Hickson-Brick *et al*. 2000, Russell *et al.* 2004, Shibata *et al*. 2005, Dyck and Lopaschuk 2006). Further to this, AMPK is involved in the cardiac hypertrophic process and acts to inhibit protein synthesis, whilst mutations for activating and inactivating AMPK are suggested to contribute to cardiac hypertrophy (Dyck and Lopaschuk 2006). Moreover, AMPK activation can also be via hormonal regulation, such as insulin, however it is unclear how insulin inhibits AMPK whilst this is hypothesised to be mediated via Akt activation and AMPK acts to inhibit insulin signalling (Kovacic *et al*. 2003, Longnus *et al*. 2005).

Activation of AMPK during a state of intracellular stress, such as ischaemic injury, requires and involves increases AMP and the activation of upstream AMPK kinases, suggesting the possible role of AMP as a control mechanism of AMPK activation within the heart (Dyck and Lopaschuk 2006). The study by Frederich and Balaschi (2002), involving a P-NMR approach investigating the calculating of AMP concentrations, demonstrated that cardiac AMPK can be activated via AMP concentrations within the low micromolar range. This suggests that lowering of AMP concentrations can be used to activate AMPK in the absence of ischaemia, such as in the response to an increased workload (Frederich and Balaschi 2002). Within the heart AMPK phosphorylation is key to controlling AMPK activation, whilst phosphorylation of threonine within the activation loop of the α1 and α2 subunit via upstream AMPK kinases is known to be a key mechanism to activate AMPK during metabolic stress (Hardie and Carling 1997, Dyck and Lopaschuk 2006). AMPK kinases LKB1 and CAMKK are identified within the heart; with CAMKK expressed at low levels and LKB1 being highly expressed in the heart, however data regarding the role of both in AMPK phosphorylation is limited (Dyck and Lopaschuk 2006, Hawley *et al*. 2003, Hawley *et al*. 2005). LKB1 is a serine/threonine kinase that is involved in several pathways including AMPK activation by responding to anoxic stress via phosphorylation of the α subunit at the Thr^{172} residue, independently of AMP concentration (Baron *et al*. 2005, Hawley *et al*. 2003, Hawley *et al*. 2005). However, it is worth noting that during events of ischaemia LKB1 acts to phosphorylate the α2 subunit and not the α1 subunit, highlighting the possible

differential regulation and physiological roles of catalytic complexes and upstream kinases (Arad *et al*. 2007, Sakamoto *et al*. 2006).

In healthy hearts large amounts of ATP are produced and sustained by mitochondrial oxidative metabolism and glycolysis. Within the heart, ATP is primarily produced via oxidation of fatty acids and pyruvate from either glycolysis or lactate, by the mitochondria (Dyck and Lopaschuk 2006). It is estimated that 10-40 % of ATP is produced via pyruvate oxidation; while 60-90 % of ATP is derived from fatty acid oxidation (Dyck and Lopaschuk 2006). AMPK can also have an important role in fatty acid and glucose metabolism and is known to regulate malonyl CoA, which acts to inhibit carnitine palmitoyl transferase (CPT)-1 on the mitochondria membrane (Dyck and Lopaschuk 2006). During events of ischaemia and ischaemic heart injury, mitochondrial oxidative metabolism decreases whilst ATP production is also decreased (Dyck and Lopaschuk 2006). During mild ischaemia energy demand is greater than the supply from mitochondrial glucose and fatty acid oxidation, resulting in glycolysis acceleration to compensate for the ATP supply (Dyck and Lopaschuk 2006). Moreover, during ischaemia ATP production is primarily via glycolysis however glycolysis becomes uncoupled from glucose oxidation, resulting in accumulation of by-products lactate and protons within cardiac cells (Dyck and Lopaschuk 2006). This results in the re-direction of ATP from myocardial contraction and towards clearing glycolytic by-products, decreasing cardiac function and efficiency (Dennis *et al*. 1991, Liu *et al*. 1996, Dyck and Lopaschuk 2006). AMPK is activated during myocardial ischaemia, occurring to restore myocyte ATP levels, and activating ATP generating pathways for glucose and fatty acid metabolism (Dyck and Lopaschuk 2006). AMPK activation results in the increase in cardiac glucose utilisation, promoting the translocation of GLUT4 to the sarcolemma of the myocyte and promoting glucose uptake, whilst also phosphorylated and activating phosphofructokinase 2, thereby phosphorylating fructose-2,6-biphosphate, stimulating glycolysis, and benefiting the heart via increasing glucose utilisation and anaerobic ATP synthesis (Russell *et al*. 2004, Li *et al*. 2005, Marsin *et al*. 2000, Young e*t al*. 1966, Merrill *et al*. 1997, Marsin *et al*. 2000).

AMPK is highly expressed within the heart and can be activated by pressure overload hypertrophy, endogenous AMPK activation was able to protect cardiac myocytes against hypoxic injury via attenuation of endoplasmic reticulum stress, the endoplasm reticulum-dependent apoptotic pathway was said to be a mechanism of hypoxic injury in cardiac myocytes (Terai *et al*. 2005, Stapleton *et al*. 1996, Hardie e*t al*. 1997, Sambandam and Lopaschuk 2003, Tian *et al*. 2001, Xing *et al*. 2003). Terai *et al*. (2005) demonstrated hypoxia activation of AMPK in cultured cardiac myocytes, whilst pretreatment with the AMPK-activator AICAR was able to further activate AMPK attenuating hypoxic injury. It has been previously indicated that hypoxia can regulate AMPK activity, with and without the presence of cytosolic AMP (Frederich *et al*, 2005, Terai *et al*. 2005). The study by Frederich *et al*, (2005) used potassium-arrested hearts perfused with varying oxygen content and demonstrated the α-subunit of Thr^{172} phosphorylation increased in the presence of oxygen concentrations ≤ 21%, whilst measured AMP was recorded at ≤ 0.3μM concertation, highlighting that oxygen concentrations ≤ 21% increased AMPK phosphorylation and activity independently of cytosolic AMP. It was mentioned by Frederich *et al*, (2005) that the hypoxic increase in AMPK activity may result from direct phosphorylation of Thr^{172} via an upstream kinase or a reduction in the $A_{0.5}$ for cytosolic AMP. Following this, AMPK has been suggested to play a role in mild ischaemia without a global change in high-energy phosphate homeostasis (Terai *et al*. 2005).

Cardioprotection by AMPK focuses on downstream targets on mitochondria, including PPAR-α and PPARγ co-activator 1α (PGC-1α) contributing to the cascade of coordinate energy metabolism within the heart, particularly as PPARs are nuclear hormone receptors that act as central regulators of cardiac fatty acid metabolism (Ingwall 2004, Barreto-Torres *et al*. 2012, Vega *et al*. 2010, Mu *et al*. 2001, Schreiber *et al*. 2003). Whilst three PPAR isoforms exist (α, β/δ and γ), PPARα was shown to be the main isoform within the heart and is the primary regulator of fatty acid metabolism (Barreto-Torres *et al*. 2012), whilst being indicative in cardioprotection (Bulhak *et al*. 2009, Yue *et al*. 2003, Ingwall 2004). PGC-1α acts as an integrator of the transcriptional network for regulating cardiac mitochondrial biogenesis, whilst also regulating genes required for the cellular uptake of fatty acids and fatty acid oxidation via activating PPARs and oestrogen-related receptors (Huss

and Kelly 2004, Kelly and Scarpulla 2004). AMPK activation results in the upregulation of mitochondrial biogenesis and increases the expression of the electron transport chain enzymes via phosphorylation of PGC-1α (Jager *et al*. 2007). Further to this, cardiac fibrosis was inhibited via AMPK activation involving PPARα activity (Fujita *et al*. 2008). The study by Fujita *et al*. (2008) demonstrated adiponectin enhanced PPARα activity though the activation of the AMPK signalling pathway in cardiac fibroblasts. The activation of AMPK was further shown to suppress Erk 1/2 activity, from this the author's concluded that adiponectin-AMPK–PPARα pathway is potentially involved in pathogenesis for cardiac fibrosis (Fujita *et al*. 2008).

1.12. Role of mTOR in Metformin cardioprotection.

As mentioned in Sections 1.9. and 1.11., Metformin is associated with cardioprotective properties by interfering with cellular pathways associated with ROS signals and DNA damage responses, cellular energy homeostasis and amino acid availability, converging in restrained mTORC1 signalling (Melnik and Schmitz 2014). mTORC1, composed of proteins mTOR, Raptor and mLST8, functions as a key regulator of cell growth and cellular proliferation, whilst Raptor acts to interact with substances for mTOC1-mediated phosphorylation (Sarbassov *et al*. 2005, DeYoung *et al*. 2007). mTORC1 is a multi-domain protein consisting of serine/threonine protein kinase domain at the C-terminus related to PI3K, within mammalian cells it is known that two functional complexes exist: mTORC1 and mTORC2 (Melnik and Schmitz 2014, Inoki *et al*. 2005, Sengupta *et al*. 2010, Laplante and Sabatini 2012). As mentioned, AMPK is a critical regulator of mTORC1, whilst the serine/threonine kinase LKB1 is considered to be the major kinase involved in phosphorylating the AMPK activation loop during energy stress (Shaw 2009, Xu *et al*. 2012, Shaw *et al*. 2005, Hardie 2007). AMPK suppresses mTORC1 via phosphorylating TSC2 and Raptor, upon activation during energy-deficient conditions and the rise in AMP levels (Gwinn *et al*. 2008, Pikiou *et al*. 2015). The inhibition activates autophagy via initiation of the Unc-51 like autophagy activating kinase (ULK-1), whilst hexokinase-II binds to mTORC1, decreasing mTORC1 activity during glucose deprivation (Egan *et al*. 2011, Shang *et al*. 2011, Roberts *et al*. 2014, Rabinowitz and White 2010).

mTORC1 is known to control the G_1/S transition and G_2/M progression of the cell cycle as well as playing a role in sensing ATP levels and ROS signals involved in regulating cell growth and proliferation (Melnik and Schmitz 2014, Wang and Proud 2009). It has been suggested that the involvement of the aberrant mTORC1 signalling is associated with the onset and progression of obesity, T2DM and certain cancers, whilst enhanced mTORC1 signalling has been shown to stimulate adipogenesis and the increased expression of key adipogenesis transcription factors PPARγ and SREBP1 (Zoncu *et al*. 2011, Dazert and Hall 2011, Cornu *et al*. 2013, Dann *et al*. 2007, Mieulet and Lamb 2010, Proud 2011, Takahara and Maeda

2013, Laplante and Sabatini 2013, Hara *et al*. 1998, Lynch *et al*. 2001, Lynch 2001, Pham *et al*. 2000, Kim and Chen 2004, Melnik and Schmitz 2014).

The attenuation of mTORC1 overexpression signalling is hypothesised to aid in the prevention of the onset of T2DM, HF and cancer. mTORC1 regulates protein and lipid synthesis, nucleoside synthesis, cell cycle progression and couples nutrient availability to cell growth and cancer (Melnik and Schmitz 2014, Hsieh *et al*. 2012, Wang and Proud 2009, Efeyan and Sabatini 2010, Ekim *et al*. 2011, Robitaille 2013, Ben Sahra *et al*. 2013, Thoreen *et al*. 2012, Clohessy *et al*. 2012). From this, the dysregulation of elements of the mTORC1 pathway have been reported in cancers, this includes PI3K amplification/mutation, loss of PTEN function and overexpression of Akt (Populo *et al*. 2012, Nardella *et al*. 2009, Vadlakonda *et al*. 2013, Mita *et al*. 2003, Barrett *et al*. 2012, Beauchamp and Platanias 2013).

ATP levels play a role in maintaining genomic stability, particularly as the mTORC1 pathway can be regulated by oxidative stress that down-regulates mTORC1 signalling (Lavin and Khanna 1999, Alexander *et al*. 2010). Metformin primarily acts on the liver; however, OCT1 is also found on cancer cell surfaces and plays an important role in antiproliferative action of Metformin in cancer cells (Segal *et al*. 2011). Upon uptake via OCT1, Metformin is believed to alter cellular energy metabolism associated with direct and indirect stimulation of AMPK in cancer cells, inhibiting mTOR and mTORC1 release (Pernicova and Korbonists 2014). Activation of LKB1 phosphorylates and activates the catalytic α-subunit of AMPK, loss of LKB1 was shown to increase mTORC1 signalling in LKB1+ mice with intestinal tumours, detected by the phosphorylation of the major downstream targets of p70 ribosomal S6 kinase and eukaryotic translation initiation factor 4B binding protein 1 (4E-BP1) (Shaw e*t al*. 2005, Zhou *et al*. 2001, Rena *et al*. 2013, Shaw *et al*. 2005).

Following the inhibition of mitochondrial complex-1 activity, Metformin was shown to enhance the expression of fibroblast growth factor 21; the increased expression as well as the inhibition of mTORC1 was associated with an increase in lifespan (Zhang *et al*. 2013, Zhang *et al*. 2012, Rallis *et al*. 2013, Mendelsohn and Larrick 2012). The induced expression of fibroblast growth factor 21 is known to be mediated via activating transcription factor 4 and is associated with mTORC1 inhibition (Zhang *et al*. 2013, Bruning *et al*. 2014). In response to stress conditions

the DNA-damage-inducible transcript 4 (DDIT4) acts to repress signalling through mTORC1 whilst also inhibiting mTORC1 signalling by stabilising the TSC1-TSC2-TBC1D7 inhibitory complex (Yoshida *et al*. 2010). p53 is required for the induction of DDIT4 and the activation of the TSC complex to inhibit mTORC1 signalling (Ellisen *et al*. 2002, DeYoung *et al*. 2008, Cam *et al*. 2014). DDIT4 acts to suppress mTORC1 signalling by releasing TSC2 from Akt-mediated association with inhibitory 14-3-3 proteins, this is said to be a physiological mechanism in response to hypoxia (DeYoung *et al*. 2008, Cam *et al*. 2014). p53 was shown to be involved in suppressing carcinogenesis via the inhibition of mTORC1, in particular p53 is a target of AMPK, with AMPK acting to induce phosphorylation of p53 and AMPK-dependent cell-cycle arrest (Akeno *et al*. 2014, Zhang *et al*. 2012, Jones *et al*. 2005). Mdmx is a negative regulator of p53, Metformin activation of AMPK results in the phosphorylation of and inactivation of Mdmx, resulting in p53 stabilisation and activation (Wade e*t al*. 2010, He *et al*. 2014). p53 induces Sestrin 1 and 2, which are known to activate AMPK and phosphorylate TCS2, resulting in the inhibition of mTORC1 (Budanov and Karin 2008). Therefore, AMPK activation, p53 activation and Sestrin 1 and 2 expressions via Metformin are described to function as a feed-forward loop to inhibit mTORC1 signalling (Hay 2011). AMPK is also a major downstream target of ATM, a member of the PI3K family and plays a role in activation of p53 (Sanli *et al*. 2010, Sun e*t al*. 2009, Fu *et al*. 2008). Inhibition of ATM was demonstrated to result in the reduction of Metformin-stimulated phosphorylation of AMPK in rat hepatoma cells, highlighting the role ATM has during Metformin administration (Melnik and Schmitz 2014, Vazquez-Martin *et al*. 2011, Lee *et al*. 2012). It has been hypothesised that Metformin could sensitise cells against further damage following the activation of the ATM-mediated DNA damage response, said to be mimicking the precancerous stimulus which acts as an intrinsic barrier against carcinogenesis (Melnik and Schmitz 2014). Further to this, Metformin is also hypothesised to function as a tissue sweeper of pre-malignant cells prior to gaining stem cell or tumour initiating properties (Melnik and Schmitz 2014, Menendez *et al*. 2011).

The exact mechanism of Metformin inhibition of guanosine triphosphate (GTP)-ases and the resulting suppression of mTORC1 signalling is still in debate (Melnik and Schmitz 2014, Kalender *et al*. 2010). It is known that proton-assisted amino acid

transporters interact with recombination activating gene (RAG)s and are required for mTORC1 activation (Melnik and Schmitz 2014, Ogmundsdottir *et al*. 2012, Goberdhan 2010). The proton-assisted amino acid transporter 1 (PAT1) has been established as the essential mediator of amino-acid-dependent mTORC1 activation in the function of the PAT1/RAG/Ragulator complex (Ogmundsdottir *et al*. 2012), the mTORC1-regulatory role of PATs said to be conserved in humans (Heublein *et al*. 2010). The activation of mTORC1 requires PAT1 and PAT4 and is elevated in PAT1-overexpressing cells (Heublein *et al*. 2010). PATs are hypothesised to modulate activity of mTORC1 by modulating the intracellular response to amino acids, as PAT1 and mTORC1 co-localise at the surface of the same intracellular compartments (Heublein *et al*. 2010). The over-expression of PAT1 enhanced the sensitivity of the mTORC1 response to amino acids in starved cells following refeeding, whilst the siRNA knock-down of the PAT1 gene inhibited mTORC1 activation, thereby highlighting that suppression of PAT1 attenuates mTORC1 signalling (Heublein *et al*. 2010). However, it was shown by Melnik and Schmitz (2014) that Metformin in excessive concentrations (10 mM) exhibited modest PAT1 inhibition of L-[^{3}H) proline uptake in Caco-2 cells, whilst only a few studies exist demonstrating the effects of biguanides on PAT4 activity, involved in the regulation of mTORC1 (Matsui and Fukuda 2013). It is worth investigating the effect of different Metformin concentrations on PAT1 and PAT4 expressing cells, to elucidate the potential inhibitory effect of Metformin on PAT/RAG/Ragulator/v-ATPase nutrisome-mediated mTORC1 signalling (Melnik and Schmitz 2014).

To conclude, Metformin administration is associated with cardioprotective properties via the inhibition of certain pathways, particularly the mTORC1-assocaited pathway. Dysregulation of the mTOR and mTORC1-associated pathways is demonstrated in certain cancers as well as being associated with increases in ROS signalling and onset of HF. Metformin acts to phosphorylate TSC2 and LKB1, inhibiting mTOR and mTORC1, as well as regulating key proteins such as ATM and p53. The importance of mTOR and mTORC1 is expanded in Section 1.13.

1.13. mTOR involvement in cancer and hypoxia.

As discussed in Section 1.12., the loss of control of mTORC1 activity is a key hallmark of malignant tumours, indicating the importance in tumourigenesis (Guertin and Sabatini 2007, DeYoung *et al*. 2007). TSC1/2 acts to inhibit the activity of mTORC1 (Hay and Sonenberg 2004), as well as functioning to integrate the cellular response to growth factor, nutrient and oxygen availability (Pan *et al*. 2004, DeYoung e*t al*. 2007). Regulation of mTORC1 is known to involve growth factor signalling, promoting mTORC1 activity via inhibition of the TSC1/2 complex (Gao and Pan 2001, Potter e*t al*. 2001, DeYoung *et al*. 2007).

TSC2 is activated via phosphorylation of the LKB1/AMPK pathway, thereby contributing towards mTORC1 inhibition during energy deprivation and long-term hypoxic stress (Corradetti *et al*. 2004, Shaw e*t al*. 2005, Liu *et al*. 2006, DeYoung *et al*. 2007). mTORC1 activity is shown to be inhibited during exposure to modest hypoxia (1 % O_2) (Arsham e*t al*. 2003) via a pathway involving the TSC1/2 complex and the REDD1 gene (Brugarolas *et al*. 2004, Reiling and Hafen 2004). Knowing that oxygen is an essential regulator of cellular metabolism, within hypoxic conditions cells are known to rapidly activate a variety of adaptive mechanisms to limit energy loss via inhibiting energy-intensive processes including protein translocation (Wouters *et al*. 2005, Liu e*t al*. 2006, DeYoung e*t al.* 2007). Is it suggested that a period of hypoxic stress occurs during tumourigenesis and that failure to suppress mTORC1 activity under hypoxia can contribute towards tumourigenesis (DeYoung *et al.* 2007). This highlights a potential role Metformin has in inhibiting and supressing mTORC1 formation, in order to suppress tumourigenesis.

1.14. Role of Adenosine and hENT1 in AMPK signalling

Studies have demonstrated the cardioprotection of Metformin to be inhibited via adenosine receptor antagonism with 8-p-sulfophenyltheophylline hydrate (8-SPT) and the equilibrative nucleoside transporter (ENT) inhibitor nitrobenzylthioinosine (NBTI), inhibiting the AMPK and PI3K/Akt pathway (Figure 10) (Paiva *et al*. 2009, Bhamra *et al*. 2008). It was demonstrated that facilitated diffusion of adenosine through the human equilibrative nucleoside transporter (hENT) to the extracellular compartment occurs and stimulates the adenosine receptor to provide cardioprotection (Paiva *et al*. 2009). The use of NBTI indicates the importance of adenosine simulation by either increased intracellular or extracellular formation of adenosine, suggesting that Metformin has intracellular adenosine concentration increasing properties (Paiva *et al*. 2009). The administration of Metformin during reperfusion resulted in the significant reduction of myocardial infarction; the effect was abolished in the presence of an AMPK-inhibitor Compound C (Paiva *et al*. 2010).

It is known that extracellular adenosine activates adenosine receptors A1, A2A, A2B, A3, and A3AR on the membrane surface of cardiac myocytes, acting to regulate pacemaker activity, producing physiological responses (Linden 2005, Rose *et al*. 2010, Mubagwa and Flemeng 2001). Levels of adenosine within the cell are regulated by the presence of and activity of nucleoside transporters found on the surface of cell membranes (Linden 2005, Rose *et al*. 2010). The influx of adenosine across the cardiac myocyte cell membrane is known to be mediated via ENT1 and is known to be expressed at higher levels in heart tissue compared to other ENT isoforms, highlighting the role of ENT1 for adenosine transport (Rose *et al*. 2010, Lu *et al*. 2004, Pennycooke e*t al*. 2001). This can be used to suggest the role of ENT1 for adenosine transport, required for AMPK and ATP production. However the use of AMPK and ENT1 signalling is still in debate, the study by Rose e*t al*. (2010) failed to find evidence towards the role of ENT1 in the modulation of AMPK signalling via exogenous added adenosine, the authors went on to suggest that the mechanism of cardioprotection may involve other pathways aside from AMPK in mice with the ENT1 gene knocked out (Rose *et al*. 2010). The study further went on

to suggest that cardioprotection was offered in the absence of ENT1 in ENT1-null transgenic mice (Rose e*t al*. 2010).

Adenosine is known to be taken into mammalian cells via ENT and concentrative nucleoside transporter (CNT) (Baldwin *et al*. 2004, Gray *et al.* 2004, Pastor-Anglada *et al*. 2004, Aymerich *et al*. 2006). ENT1 and CNT2 are primarily responsible for this process, the expression of both transporters is said to be widespread and have demonstrated a K_m value for adenosine in the physiological range of 40 µM for ENT1 and 8 µM for CNT2 (Wang *et al*. 1997, Ward *et al*. 2000). Adenosine is reported to be present in peripheral blood at concentrations of 0.3-1 µM, moreover the concentration is considered to be higher in the intestinal lumen and the portal vein (Lasey *et al*. 1998, Pasini *et al*. 1996, Saadjian *et al*. 2002). Further to this, adenosine is known to be produced via ectonucleotidase action on nucleotides, contributing to higher local nucleoside concentrations (Che *et al*. 1997, Roman and Fritz 1999).

The adenosine receptor system is said to enhance resistance to ischaemic injury under cellular and metabolic stress within the heart, the purine nucleoside adenosine functions to mitigate myocardial damage from ischaemia-reperfusion injury (Peart and Headrick 2007). Under ischaemic injury endogenous levels of adenosine are said to increase rapidly to mediate a response, adenosine is formed following dephophorylation of 5'AMP via intracellular and extracellular 5'-nucleotidases and from S-adenosylhomocysteine and is taken into cells by the facilitative ENT (Headrick *et al*. 2003). Within cells the adenosine is de-aminated via adenosine deaminase or rephosphorylated to 5'AMP by adenosine kinase (Headrick *et al*. 2003). Small changes in intracellular AMP concentrations have demonstrated a modulating effect on AMPK activity in a synergistic manner (Aymerich *et al*. 2006). With the knowledge that adenosine is taken into mammalian cells via ENT, it was demonstrated that extracellular adenosine was able to activate AMPK within epithelial cells, this was said to involve adenosine transport and resulted in an increase in the AMPK intracellular pool (Aymerich *et al*. 2006). Using this information, it can be suggested that inhibiting extracellular adenosine, required for AMPK activation, would result in the decrease or inactivation of AMPK.

The study by Aymerich *et al*. (2006) demonstrated that the prevention of adenosine phosphorylation resulted in an increase yield in AMP signalling, using the inhibitor of adenosine kinase 5'-iodotubercidin (5'-ITU) following pre-treatment of IEC-6 cells. The study further demonstrated the inhibition of increased intracellular AMP concentrations after adenosine addition, and a marked decrease in ADP concentration, the authors concluded that the generation of AMP from extracellular adenosine was required for AMPK activation (Aymerich *et al*. 2006). Further to this, the role of ENT-type transporters on the adenosine-triggered activation of AMPK was investigated by Aymerich *et al*. (2006) using 1 µM NBTI, prior to adenosine addition to cultured cells. The authors concluded that the ENT-related transport function was inhibited by approximately 80 % (Aymerich *et al*. 2006). The authors concluded that extracellular adenosine was able to activate AMPK via a mechanism requiring adenosine transport, resulting in an increase in the AMP intracellular pool (Aymerich *et al*. 2006). The same study further demonstrated that partial inhibition of extracellular adenosine, via the inhibition of CNT2, resulted in a significant blockade of the AMPK pathway and by using 1 µM adenosine a consistent and significant increase in AMPK was achieved (Aymerich *et al*. 2006). The addition of extracellular adenosine was shown to enhance ACC phosphorylation, which was otherwise abolished following CNT2 inhibition (Aymerich *et al*. 2006).

Endogenous adenosine demonstrated cardioprotective properties during ischaemia-reperfusion studies, reducing the infarct size and delaying the onset of ischaemic contracture by reducing the rate of ATP catabolism (Ely and Berne 1992). Adenosine further demonstrated cardioprotection involving cardiac myocytes via inhibiting platelet aggregation, preventing adhesion of inflammatory cells to the endothelium and reducing the production of superoxide via neutrophils (Ely and Berne 1992, Mubagwa *et al*. 1996). The administration of exogenous adenosine is said to be effective in ischaemic and pharmacological preconditioning (Wang *et al*. 2002). However, it was said that the uses of adenosine receptor agonists are difficult to use in clinical settings due to complicated side effects of drugs on extra cardiac tissue and by progressive desensitisation of receptors to high doses of adenosine (Maddock e*t al*. 2002). The high level of extracellular adenosine during ischaemia can potentially reduce the efficacy of adenosine receptor agonists (Van der Heide and Reimer 1996, Matherne *et al*. 1997).

Figure 10: The proposed mechanism of Metformin-induced cardioprotection with the involvement of adenosine for the activation of AMPK (Bromage and Yellon 2015). *Key: Ado: Adenosine.*

The study by Maddock *et al*. (2002) demonstrated that the activation of adenosine A3 receptors, after administrating the A3 receptor agonist 2-CL-IB-MECA, was able to reduce the infarct percentage increase after ischaemia-reperfusion in isolated perfused rat hearts, whilst further exerting an anti-apoptotic and anti-necrotic effect in cardiac myocytes. Adenosine A3 receptor activation was hypothesised to mediate both cell protection and cell death depending on the degree of receptor activation, moreover the threshold for A3 mediated decrease in apoptotic injury is said to be mild (Maddock e*t al*. 2002, Abbracchio and Cattabeni 1999, Gao *et al*. 2001, Shneyvays e*t al*. 2001, Von *et al*. 2001, Yao *et al*. 1997). However, concentrations of 2-CI-IB-MECA greater than 10 μM are said to trigger necrosis or apoptosis of cell death (Maddock *et al*. 2002, Abbracchio and Cattabeni 1999, Shneyvays *et al*. 2000, Shneyvays *et al*. 1998). From this, the concentration of 1 nM for A3 agonism was said to be ideal, particularly as higher concentrations of 30 and 100 nM were said to not demonstrate a reduction in infarct percentage following ischaemia-

reperfusion of isolated rat hearts, whilst concentrations below 1 nM were also said to not demonstrate any reduction in infarct percentage (Maddock *et al*. 2002). This was further stated to demonstrate similar profiles of apoptosis and necrosis of rat cardiac myocytes (Maddock e*t al*. 2002). With this information regarding the role of adenosine signalling in cardioprotection, Metformin was demonstrated to reduce ischaemic heart damage via adenosine receptor activation (Paiva *et al*. 2009).

Studies have also demonstrated that Metformin can increase adenosine signalling in *in-vivo* and *ex-vivo* contractile models involving penile strips from high-fat diet-induced animal, of which in-vivo and ex-vivo administration of Metformin resulted in an increase in adenosine receptor relaxation via the interference of NO and increase in adenosine signalling (Vignozzi *et al*. 2014). However, a blinded-endpoint study involving healthy middle-aged participants failed to demonstrate if Metformin was able to protect against endothelial ischaemia–reperfusion injury (El Messaoudi *et al*. 2014). However, it must be noted that the study involved the use of forearm ischaemia and Metformin was administered as a form of short-term pre-treatment and not during the event of ischaemia or reperfusion. The authors mentioned the hypothesis was driven following studies by Paiva *et al*. (2009), Bhamra *et al*. (2008), Calvert *et al*. (2008) and Gundewar *et al*. (2009), however only Calvert *et al*. (2008) of the aforementioned studies involved pre-treatment of Metformin prior to ischaemia, the others focussed on Metformin administration throughout the course of ischaemia or during reperfusion following the initiation of a form of ischaemia. Moreover, unlike El Messaoudi *et al*. (2014), Calvert *et al*. (2008) demonstrated that Metformin was able to limit the extent of myocardial damage following pre-treatment of Metformin in non-diabetic mice hearts. Using this, it can be suggested that Metformin administration during an event of ischaemia could result in the prevention of myocardial damage, via the activation of the AMPK signalling pathway.

1.15. Ex-vivo modelling for drug-induced ischaemic heart disease.

Investigating Sunitinib-induced cardiotoxicity and Metformin-induced cardioprotection can be performed using the Langendorff technique for both normoxic and ischaemia-based experiments, using animal hearts. The Langendorff system can be used to stimulate the effects of perfusion of compound into the heart, the technique allows the heart to be perfused by cannulating the aorta whilst perfusion buffer flows retrogradely down the aorta, opposite normal physiological flow (Bell *et al*. 2011). This can allow for both Sunitinib and Metformin to be retrogradely perfused directly into the heart. During perfusion, haemodynamic parameters such as left ventricular developed pressure (LVDP), heart rate (HR) and coronary flow (CF) as well as further parameters can be measured (Bell *et al*. 2011). Furthermore, isolated heart tissue following Langendorff perfusion can be used to measure infarct percentage by using the 2, 3, 5 triphenyltetrazolium chloride (TTC) staining technique. TTC is used to visually detect infarcted area of the animal heart following Langendorff perfusion, TTC is reduced to triphenyltetrazolium formazan (TTF), TTF allows for the distinguishing between intact myocardium (brick-red colour) where dehydrogenase activity is preserved, and infarct myocardium (pale colour) where enzymes are inactivated or reduced (Kokimoto *et al*. 2013).

The Langendorff technique and TCC staining will allow for the measuring of drug–induced changes to haemodynamic parameters as well as measuring potential drug–induced damage to the heart. The Langendorff system is described as a highly reproducible and neither time-consuming or technically demanding (Skzypiec-Spring *et al*. 2006). However, when using the Langendorff system, the system needs to be tailored appropriately for the desired organ used and the animal model. Perfusion pressure is typically between 70 and 80 mmHg as described by Bell *et al*. (2011), whilst coronary flow remains dependent on the size of the heart being used, typical figures are 2 ml/min for mouse, 20 ml/min for rat and 40 ml/min for rabbit (Bell *et al*. 2011). The column of perfusate is above a certain height above the perfusion cannula tip, therefore pressure at the cannula should be the same as throughout the Langendorff apparatus, however once flow is established restriction to the delivery circuit, including the diameter of tubing, coil heat exchanges, tubing connectors, sintered glass in line filters and the presence of air bubbles or debris,

can lead to a significant drop in perfusion pressure (Bell *et al*. 2011). In particular, cannula design is 3 mm in outer diameter when using rat hearts (Bell *et al*. 2011). Isolated hearts are perfused with a physiological salt solution containing bicarbonate similar to the ionic content of plasma originally defined by Krebs and Henseleit following replacement of animal blood as used originally by Langendorff and must be delivered at 37 ^{o}C whilst continuously gassed with 5 % CO_2 and 9 5% O_2, pH 7.4 (Langendorff 1898, Taegtmeyer 1995, Skzypiec-Spring *et al*. 2006, Doring 1990, Sutherland and Hearse 2000, Ytrehus 2000). Following this, we propose the use of the Langendorff system to monitor the potential cardiotoxic and cardioprotective properties during drug perfusion.

1.16. Aims and objectives.

Taking into consideration of all information discussed in Section 1, we aim to investigate the following aims and hypothesis:

1. Sunitinib malate will demonstrate properties of cardiotoxicity when administered at a clinical dose as highlighted by existing research studies, we propose that the anti-diabetic agent Metformin hydrochloride will demonstrate potential properties of cardioprotection when administered together with Sunitinib.
2. Sunitinib-induced cardiotoxicity may result from the inhibition of AMPK inactivation, equally Metformin-induced cardioprotection may result from the activation of AMPK. This will be demonstrated using the AMPK-associated inhibitor S-4-Nitrobenzyl)-6-thioinosine (NBTI), administered with Metformin and Sunitinib.
3. We aim to investigate the anti-cancer properties of Metformin and Sunitinib co-administration in cancer cell lines of HepG2 liver cancer and HL60 human leukaemia cells, as potential adjunctive treatment.

Chapter Two: Materials and Methods.

2.1. Drug concentration rationale and introduction

Metformin at a 50 µM concentration was selected to be used in the *ex-vivo* Langendorff model and the *in-vitro* cancer cell-based models. Metformin administration at very high concentrations (5 mM) was shown to induce apoptotic death (An *et al*. 2006) and therefore maximum plasma concentration of Metformin administration was recommended to be 100 µM (Wood *et al*. 1996). Taking this into consideration the cardioprotective properties of Metformin were demonstrated at 50 µM (Bhamra *et al*. 2008, Paiva *et al*. 2009). The use of PI3K inhibitors further demonstrated Metformin's cardioprotective effects to be independent of hypoglycaemic and insulin-sensitising effects, in isolated perfused rat hearts and cardiac myocytes (Bhamra *et al*. 2008). The *ex-vivo* concentration of 50 µM is believed to be slightly greater than the average plasma concentration after administration of a single dose of 850 mg in patients (Sambol *et al*. 1996). Very few studies exist regarding the use of Metformin administration alone in normoxic conditions of the Langendorff system without use of prior-induced injury or ischaemia-reperfusion; however existing ischaemia-reperfusion studies have used Metformin at concentrations of 25 µM, 50 µM and 75 µM in isolated perfused rat heart models. Metformin at concentrations of 50 µM and 75 µM demonstrated a reduction in infarct size when administered at reperfusion following ischaemia, however 25 µM Metformin failed to demonstrate a cardioprotective effect (Bhamra *et al*. 2008). Metformin is indicated to achieve a plasma concentration of 10-20 µM~~l~~ in patients, Hawley *et al*. (2002) was able to demonstrate a significant activation of AMPK in H4IIE cells at a Metformin concentration of 50 µM, whilst activation was said to be two-fold greater following 72 hours of incubation. It was said that higher doses of Metformin are not required for maximal AMPK activation to achieve a beneficial therapeutic effect (Hawley *et al*. 2002). Furthermore, in patients, greater doses of Metformin are said to result in gastrointestinal side effects, whilst also posing a risk for lactic acidosis, possibly resulting from the compound acting as an inhibitor of the respiratory chain at higher concentrations (Hawley *et al*. 2002, Owen *et al*. 2000).

The concentration of 1 µM for Sunitinib was selected to be used in the *ex-vivo* Langendorff model and the *in-vitro* cancer cell-based models. Sunitinib is reported to achieve a steady state blood concentration at 50 ng/ml in cancer patients, corresponding to 0.09-1 µM (Goodman *et al*. 2007, Henderson e*t al*. 2013). Furthermore, Sunitinib drug solution was made to achieve a final dimethyl-sulfoxide (DMSO) concentration of 0.1 % or lower in all solutions, it is believed that there are no changes in contractility in cardiac *ex-vivo* experiments with <0.1 % DMSO in Krebs Henseleit (KH) bicarbonate buffer (Henderson e*t al*. 2013).

The concentration of 1 µM for NBTI was selected to be used in the animal and cancer cell-based models. As mentioned in Section 1.14., facilitated diffusion of adenosine through the hENT was shown to stimulate the adenosine receptor to provide cardioprotection via the activation of AMPK (Paiva *et al*. 2009). Metformin's cardioprotective properties, via adenosine receptor stimulation was demonstrated after adenosine receptor stimulation was abolished via ENT inhibitor NBTI (1 µM), inhibiting AMPK and the PI3K/Akt pathway (Paiva *et al*. 2009, Bhamra *et al*. 2008). Moreover, similar results were shown at a concentration of 1 µM by Aymerich *et al*. (2006) and Rose e*t al*. (2010).

2.2.1. Materials

Metformin hydrochloride (500 mg, MW 165.62 mg/mM) was purchased from Sigma Aldrich, Gillingham, UK. 500 mg of Metformin was dissolved in 30.184 ml ultra-pure RO water in order to bring to a final concentration of 0.1 M stock solution. The Metformin stock solution was stored at -20^{o}C.

Sunitinib Maleate (25 mg, MW 532.56 mg/mM) was purchased from Sigma Aldrich, Gillingham, UK. The 25 mg of Sunitinib was dissolved in 4.69 ml of DMSO in order to bring to a final concentration of 0.01 M stock solution. The Sunitinib stock solution was stored at -20^{o}C and care was taken to avoid exposure to light and UV radiation.

NBTI (S-4-Nitrobenzyl)-6-thioinosine, 100 mg, MW 419.41 mg/mM) was purchased from Sigma Aldrich, Gillingham, UK. NBTI was dissolved in 23.84 ml DMSO in order to bring to a final concentration of 0.01 M stock solution. The NBTI stock solution was stored at -20^{o}C.

AMPKα, Phospho-AMPKα (Thr^{172}), GAPDH, and mAb Anti-rabbit antibodies were purchased Cell Signalling Technology® New England BioLabs, Hitchin, UK. Vials were aliquoted and stored at -20 ^{o}C. Mini-PROTEAN® TGX™ Precast polyacrylamide gels (4-10 %, mini-protein Stain-Free), and Trans-Blot® Turbo™ Midi PDVF Transfer Pack membranes were purchased form Bio-Rad, UK, and stored at 4^{o}C.

Penicillin-Streptomycin Solution (x100 concentration), Trypsin with EDTA (x10 concentration) and L-Glutamine (200 mM, x100 concentration) were all purchased from LabTech International Ltd, East Sussex, United Kingdom. Foetal Bovine Serum (FBS, South American origin) and Dulbecco's Modified Eagle Medium (DMEM) with high glucose and Sodium Pyruvate w/o HEPES were all purchased from ThermoFisher Life Technologies Ltd, Paisley, United Kingdom.

3-(-4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) powder was purchased from Sigma Aldrich, Gillingham, UK.

Remaining reagents were standard laboratory reagents from Sigma Aldrich and Fisher Scientific.

2.2.2. Animals and cells.

Experiments were performed using Sprague-Dawley (SD) rats (2-3-month male, 345-375 g b/w). Animals were obtained from Charles River research laboratories, Margate, UK. Experiments received ethical approval from Coventry University and all experiments were conducted in accordance with the Animal (Scientific Procedure) Act 1986 and Coventry University standards. SD rats were selected for use due to general purpose model and based on previous worked carried out at Coventry University by Sandhu *et al*. 2017, Cooper *et al*. 2018 and Maddock *et al*. 2002.

Experiments were performed using HepG2 and HL60 cell lines acquired from The American Type Culture Centre (ATCC) brought up from the Coventry University stock. Experiments received ethical approval from Coventry University and all experiments were conducted in accordance with the Animal (Scientific Procedure) Act 1986 and Coventry University standards.

As Sunitinib is currently used in studies for the treatment of acute myeloid leukaemia, the HL60 cell line was deemed as a suitable model to investigate Sunitinib's anti-proliferative properties. As described by ATCC, the HL60 cell line is described as a peripheral blood leukocyte promyelocytic cell line obtained from leukapheresis from a 36-year-old Caucasian female with acute promyelocytic leukaemia. The HepG2 liver cancer cell line, derived liver tissue of a 15-year old male with hepatocellular carcinoma, was used to investigate the potential anti-proliferative properties with Metformin. Moreover, the HepG2 cell line was deemed suitable for a comparison study with HL60 cells based on the use with numerous studies involving hepatotoxicity.

2.3.1. Langendorff experiment and isolated perfused heart preparation– normoxic study.

Animals were sacrificed by cerebral dislocation, a schedule one home office procedure in accordance with The Guidance on the Operation of the Animals 1986. Hearts were rapidly isolated and placed into ice cold 4^{o}C KH bicarbonate buffer. Hearts were immediately mounted onto the langendorff system, cannulated via the aorta with a 1.4 mm inner diameter, whilst being retrogradely perused with KH buffer. KH buffer was prepared in 10 litres ultra-pure Millipore/RO water containing (mM) NaCl 118.5, $NaHCO_3$ 25, KCL 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.7, glucose 11.

KH buffer was gassed with 95 % O_2 and 5 % CO_2 and maintained at 37 ± 1 ^{o}C and pH 7.4 ± 0.2 at all times. The left atrium was trimmed away, and the latex isovolumic balloon was carefully introduced into the left ventricle and inflated up to 5–10 mmHg end diastolic pressure. *Ex-vivo* rat hearts were equilibrated and stabilised for 10–20 minutes prior to starting the experiment. Functional recording was conducted using the physiological pressure transducer connected to a bridge amp and Powerlab (AD Instruments Ltd. Chalgrove, UK). LVDP, HR and CF were measured at regular intervals of 5 minutes for 20 minutes during stabilisation, intervals of 5 minutes for 35 minutes during drug perfusion, and intervals of 15 minutes for 120 minutes (155 minutes total drug perfusion) (Figure 11).

Normoxic study involved the administration of Sunitinib at 1 µM concentration ± Metformin at 50 µM concentration ± NBTI at 1 µM concentration following 20 minutes of stabilisation for each heart during the 155 minute drug perfusion (Figure 11), (refer to Section 2.1. for drug rationale). Control study was carried out without the administration either Sunitinib or Metformin or NBTI. Hearts were divided into 2 groups for either TTC staining or Western blot analysis.

2.3.2. Triphenyl-tetrazolium chloride staining.

Following Langendorff perfusion, Section 2.3.1., hearts were dismounted and were weighed and frozen at -20 ^{o}C. Frozen hearts were sliced into 2mm thick transverse sections before being incubated in TTC solution (1% in phosphate buffer) at 37 ^{o}C for 10–12 minutes and fixed in 10 % formalin for a minimum of 4 hours. Heart slices were monitored for discolouration. Risk zone and infarct area were traced onto acetate sheets. Computerised Imagetool^{TM} software was used to analyse the percentage of infarct tissue. Infarct size was normalised to the total area of each heart slice.

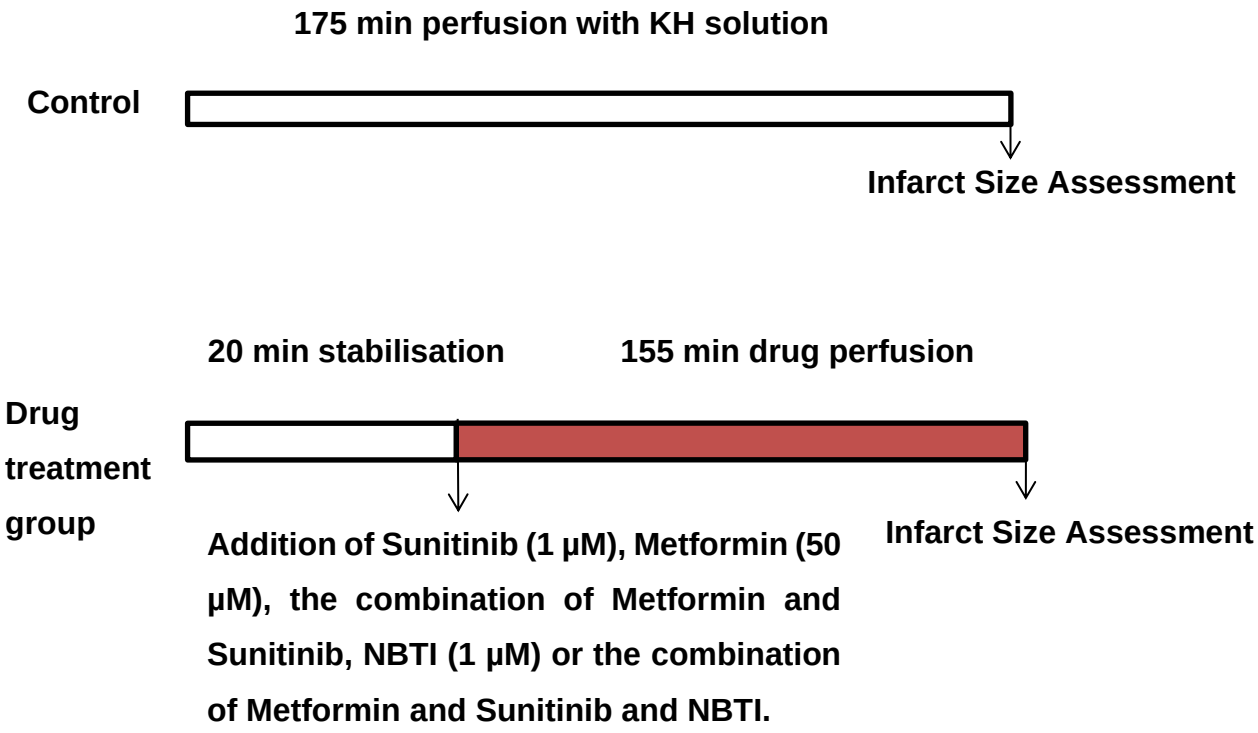

Figure 11: The treatment protocol used for the normoxic study for infarct size assessment for the Langendorff experiment.

2.4. Western blot analysis SDS Page.

Following Langendorff perfusion, Section 2.3.1., hearts were dismounted and left atrium coronary organ tissue from the normoxic study was snap-frozen in liquid nitrogen. Tissue was homogenised in Protein Lysis Buffer (100 mM Tris-Base, 1 mM EDTA, 10 % SDS, 0.1 M NaCl, 2 mM Sodium Pyruvate, 2 mM NaF, 2 mM β-Glycerophosphate, 1 protease inhibitor cocktail tablet per 100 ml, 1 PhosSTOP™ tablet per 10 ml, ultra-pure RO water), using the homogeniser (IKA® T 25 ULTRA-TURRAX).

A total of 60 µg of protein concentration was determined using Pierce™ BCA protein assay kit (ThermoFisher Scientific). Required quantities of each sample were mixed 1:1 with Sample Buffer (1 M Tris-HCl, pH 6.8, SDS, glycerol, β-Mercapto-ethanol, Bromophenol blue, ultra-pure RO water) heated to 100^{o}C for 10 minutes. Samples were diluted with the required quantity of Sample Buffer to achieve a 60 µg of protein concentration in a 15 µl well volume.

Samples were loaded onto Mini-PROTEAN® TGX™ stain-free Gel (BioRad, USA) and run for approximately 1 hour at 130 V, following protocol optimisation. Gels were transferred onto membrane via electrophoresis with Tran-Blot Turbo (BioRad, USA). Immunoblots were incubated for 1 hour with blocking buffer (5 % milk powder, x1 concentration TBST). Immunoblots were analysed using primary antibodies: Phosphorylated AMPKα (p-AMPK, Thr^{172}), total AMPKα and house-keeping protein GAPDH for overnight incubation in 4 ^{o}C, using a tube roller/orbital shaker. Immunoblots were exposed for 1 hour to specific secondary antibodies (Rabbit mAb). Proteins were visualised using enhanced chemiluminescence detection kit Super Signal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, USA). Images were taken with ChemiDoc MP System (BioRad, USA). Density was analysed with BioRad software QuantityOne. Analysis of p-AMPK protein receptor bands was normalised to AMPKα (total AMPK) and GAPDH and quantified using computerised Imagetool™ software analysis.

2.5. Methylthiazolydiphenyl-tetrazolium bromide assay for HepG2 and HL60 cell viability

The methylthiazolydiphenyl-tetrazolium bromide (MTT) assay with 3-(-4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was performed using HepG2 and HL60 cancer cells for control and treatment group: Metformin 6 µM, 10 µM, 30 µM, 60 µM, 100 µM, 300 µM, 600 µM, 1000µM, Sunitinib 0.1 µM, 0.3 µM, 1 µM, 6 µM, 10 µM, 30 µM, 60 µM and 100 µM, NBTI 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM and 100 µM. Co-administration (co-treatment) of Sunitinib 0.1-100 µM was carried out with 50 µM Metformin, co-treatment and NBTI was carried out using Sunitinib 0.1-100 µM, 50 µM Metformin and 1 µM NBTI.

HepG2 and HL60 cells were investigated for assessing cell metabolic activity including nicotinamide adenine dinucleotide phosphate (NADPH)–dependent cellular oxidoreductase enzymes to reduce tetrazolium dye MTT to the insoluble formazan product.

Cancer cells were stored in a T75 cm^2 flask containing 12 cm^2 DMEM or RPMI cell growth media. Plates containing HepG2 cells were incubated at 37 ^oC, 5 % CO_2 for 48 hours, to allow attachment >60 % confluency. Plates containing HL60 cells were incubated at 37 ^oC, 5 % CO_2 for 24 hours to allow for >60 % confluency

Plates were incubated with Sunitinib 0.1-100 µM concentration or Metformin 0.1-1000 µM concentration or NBTI 0.1-100 µM concentration at 37 ^oC, 5 % CO_2 for 24 hours. Following incubation with compounds, media was aspirated off and replaced with fresh media and 50 µl MTT media (concentration 5 mg/ml final concentration) for a total of 100 µl. The plate was incubated for 6 hours minimum at 37^oC, 5% CO_2. MTT-containing media was aspirated gently and replaced with 100 µl of DMSO. Plates were read on microtiter plate reader at 595 nm/492 nm, reference 690 nm. EC_{50} was generated from the dose-response curve for each sample using GRAPH PAD PRISM software.

For HepG2 cells, media from cell culture flask was decanted into a 30 cm^2 universal bottle; remaining cells were washed with 5 ml sterile Phosphate buffered saline (PBS) x1 concentration, cell suspension was left for 2 minutes with the flask being agitated gently at intervals during incubation. PBS, from cell suspension, was

discarded into a waste beaker. 2 ml Trypsin/EDTA solution, at x2 concentration, was added to the flask containing cells. Cells were incubated for 3–5 minutes at 37 ^{o}C, 5 % CO_2. Cell detachment was observed and recorded. Following cell layer detachment, 8-10 ml DMEM cell growth medium was added. Cell suspension was pipetted up and down, approximately 10 times, to dislodge cell clumps. Cells were centrifuged at 2,000–2,500 rpm for 5 minutes to remove dead cells, creating a visible pellet.

For HL60 cells, the cell suspension with media from cell culture flask was decanted into a 30 cm^2 universal bottle. Cells were centrifuged at 2,000-2,500 rpm for 5 minutes to remove dead cells, creating a visible pellet.

Supernatant was discarded into a waste beaker; the remaining visible pellet was resuspended in 10 ml fresh DMEM or RPMI cell growth medium. Approximately 10 µl of cell suspension was transferred to the haemocytometer to determine the approximate total cell count with Trypan blue (1:1 dilution). Live/viable cells were characterised as “white” in colour, dead/non-viable cells were distinguished as blue/dark blue in colour due to cell permeability resulting in the absorption of the Trypan blue dye.

Viability (%) was determined as the mean live cells/mean total cells or cells/cm^3 x 100 = Mean viable cell number per corner x 10^4. Calculation was carried out to produce 5x10^3 cells/ml per well of a 96-well plate.

HepG2 cancer cells were plated 5x10^3 cells/well by adding 100 µl of the 5x10^3 cells/ml cell suspension to each well of the 96-well tissue culture plate. Plate was incubated at 37^{o}C, 5% CO_2 for 48 hours to allow attachment and 40~~%~~-60 % confluency. DMEM Media was aspirated off and replaced with fresh media (100 µl) containing either Sunitinib at 0.1 µM, 0.3 µM, 1 µM, 6 µM, 10 µM, 30 µM, 60 µM and 100 µM concentration or Metformin at 0.1 µM, 0.5 µM, 1 µM, 5 µM, 6 µM, 10 µM, 30 µM, 50 µM, 60 µM, 100 µM, 300 µM, 500 µM, 600 µM and 1000 µM concentration or NBTI at 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM and 100 µM concentration. 2 rows were used as normoxic controls.

HL60 cancer cells were plated 5x10^3 cells/well by adding 100 µl of the 5x10^3 cells/ml cell suspension to each well of the 96-well tissue culture plate. Cells were

immediately incubated with either Sunitinib at 0.1 µM, 0.3 µM, 1 µM, 6 µM, 10 µM, 30 µM, 60 µM and 100 µM concentration or Metformin at 0.1 µM, 0.5 µM, 1 µM, 5 µM, 6 µM, 10 µM, 30 µM, 60 µM, 100 µM, 300 µM, 600 µM and 1000 µM concentration, or NBTI at 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM, 25 µM, 50 µM and 100 µM concentration. 2 rows were used as normoxic controls. Concentrations 0.1 µM, 0.5 µM, 1 µM and 5 µM Metformin not presented in Section 5.1.-5.5.

Plates were incubated at 37^{o}C, 5 % CO_2 for 24 hours with drug treatment. Following incubation with compounds, media was aspirated off and replaced with fresh media and 50 µl MTT media (concentration 5 mg/ml in PBS) for a total of 100 µl in each well. The plate was incubated for 6 hours minimum at 37^{o}C, 5 % CO_2. MTT-containing media was removed gently, and samples were solubilised in 100 µl of DMSO. Plates containing purple formazan dye, converted from MTT by visible cells, were read and quantified on microtiter plate reader at 595 nm/492 nm, reference 690 nm. Treatment groups were standardised to vehicle control for each plate. EC_{50} was generated from the dose-response curve for each sample using PRISM software.

2.6.1. Cardiac myocyte isolation.

SD rat (2-3 month old male, 345–375 g b/w, Charles River research laboratories, Margate, UK) left ventricular cardiac myocytes were isolated by conventional enzymatic dissociation (Maddock *et al*. 2012). Animals were sacrificed by central dislocation, following appropriate supervision and following Coventry University standards.

Hearts were immediately isolated and mounted onto a modified langendorff apparatus. Hearts were perfused with modified KH bicarbonate buffer for 5 minutes followed by 8 minutes of perfusion with collagenase buffer. Modified KH buffer was prepared in 1 litre Millipore/RO water containing (mM) NaCl 116, KCl 5.4, $MgSO_4 7H_2O$ 0.4, Glucose 10, Taurine 20, Pyruvate 5, $NaHCO_3$, KH_2PO_4. Modified KH bicarbonate buffer was oxygenated with 95 % O_2 and 5 % CO_2 and maintained at 37 ^oC (pH 7.4). Collagenase buffer was prepared at pH 7.4 in 65 ml of modified KH buffer containing 0.03 g of 1 mg/ml Collagenase and 2.2 µl of 1 M $CaCl_2$.

During perfusion with collagenase buffer, effluent was collected and reused. After perfusion with collagenase, the heart was removed, and the atrium was cut away. Ventricles were teased apart and incubated with collagenase buffer in the sterile falcon tube (1) before being suspended. Solution from tube (1) was transferred to a sterile falcon tube (2) and centrifuged at 400 rpm for 2 minutes. Fresh collagenase was added to tube (1) (containing the existing heart tissue) and suspended. The tissue suspension containing digestion buffer, from tube (1), was aspired and was passed through a nylon mesh into a sterile falcon tube before being centrifuged at 400 rpm for 2 minutes. The supernatant from each tube (1) and (2) was removed using a sterile pipette. The remaining pellet was redistributed in 25 ml restoration buffer. Restoration buffer was prepared in 200 ml of modified KH buffer at pH 7.4, containing 2 g of 1 % Bovine Serum Albumin, 2 ml of 1 % pen-strep, 0.131 g creatine and 100 µl $CaCl_2$ of 100 mM $CaCl_2$ (Hussain *et al.* 2014). Calcium concentration was gradually brought back to 1.25 mM before myocyte viability was assessed under light microscopy. Solution was discarded if cardiac myocyte viability was judged to be below 70 %.

2.6.2. Cardiac myocyte isolation-Trypan blue staining.

Following successful cardiac myocyte isolation, Section 2.6.1., cardiac myocytes were seeded into a 6 well plate. Sunitinib 1 µM ± Metformin 50 µM ± NBTI 1 µM were added to each appropriate well in with vehicle control. Cardiac myocyte cell suspension was added to each appropriate well making each well final volume 1 ml. Cells were incubated for 4 hours in the normoxic condition chamber (37 ^{o}C) before undergoing trypan blue (0.4 % w/v in 1x PBS filtered, 1:1 dilution, 2x concentration) staining for live cell population counts.

2.7. Statistical Analysis.

The data were expressed as mean ± SEM. LVDP, HR and CF were assessed for the statistical difference ($p<0.05$) using one-way analysis of variance (ANOVA) with LSD post hoc tests for each specific time point. The use of the two-way repeated measures (multi-parametric) ANOVA with Tukey HSD post hoc tests was used to assess the statistical significance ($p<0.05$) between time and time against treatment group for HR, LVDP and CF. The infarct size data was tested for group significant differences ($p<0.05$) using one-way ANOVA with LSD post hoc tests. p-AMPKα expression was quantified against AMPKα and GAPDH expression and assessed for statistical difference ($p<0.05$) using one-way ANOVA with LSD post hoc test.

Chapter Three: Metformin Attenuates Sunitinib-Induced Cardiotoxicity in Sprague-Dawley Rat Hearts using the Langendorff System.

Data and contents in this chapter were published / presented as following:

Conferences

- British Toxicology Society Annual Conference (2016), Manchester, England.
- Safety Pharmacology Society Annual Conference (2016), Vancouver, Canada.

Abstract and poster presentation

- *Early Studies Assessing the Cardioprotective and Anticancer Properties of Metformin during Sunitinib-Induced Cytotoxicity,* Safety Pharmacology Society Annual Conference 2016, Vancouver, Canada.
- *Early Studies Assessing the Cardioprotective Properties of Metformin during Sunitinib-Induced Cardiotoxicity,* British Toxicology Society Annual Conference 2016, Manchester, England.

Oral presentation

- *Early Studies Assessing the Cardioprotective Properties of Metformin during Sunitinib-Induced Cardiotoxicity,* British Toxicology Society Annual Conference 2016, Manchester, England.

3.1. Introduction

As discussed in Chapter 1, drug-induced cardiotoxicity, involving anticancer agents, has been defined as toxicity of the heart and vascular system through the adverse side-effects (Albini *et al.* 2010). Drug-induced cardiotoxicity can be attributed to one or more of the following: cardiomyopathy as a reduction in LVEF, symptoms associated with HF, signs associated with HF including S3 gallop, tachycardia or both, and reduction in LVEF from baseline in the range of less than or equal to 5 % to less than 55 % with symptoms of HF (Albini *et al.* 2010). This definition was further updated to include subclinical cardiovascular damage associated in response to chemotherapy agents (Albini *et al.* 2010). An example of this is involves tyrosine-kinase inhibitors such as Imatinib mesylate (Gleevec) used for the treatment of chronic myeloid leukaemia; subclinical cardiovascular damage was demonstrated in patients developing congestive HF and LV dysfunction, with similar effects demonstrated in mice following Imatinib treatment (Kerkela *et al*. 2007). Moreover, drug-induced toxicity may arise years after the end drug usage (Ewer and Ewer 2010), Maharsy *et al*. (2014) demonstrated the potential for Imatinib to cause a deleterious effect in cardiac myocytes in a time, dose and age dependent manner, highlighting the importance in demonstrating the effects of drug-induced toxicity in a time-dependent and long–term manner.

The multi-TKI Sunitinib is associated with incidences of a decline in LVEF however the effect was reversible upon drug discontinuation (Motzer *et al*. 2007). The exact mechanism of Sunitinib-induced cardiotoxicity is not fully established. Sunitinib binds to VEGFR and PDGFR with both receptors being associated with a key role in renal-cell carcinoma pathogenesis (Abrams *et al*. 2003, Mendel *et al*. 2003, O'Farrell *et al*. 2003). ATP is an area of particular interest in regard to drug-induced cardiotoxicity. Multi-TKIs have been reported to bind to and occupy the kinase ATP-binding sites as highlighted in the reviews by Krause and Van Etten (2005) and Roskoski (2016), a mechanism that is successful in inhibiting abnormally high kinase activity and abnormal cell proliferation in many forms of cancer. However, off-target effects as suggested by Alameddine *et* al. (2015) result in binding to PDGFR sites of cardiac myocytes within the heart, contributing to the limiting of ATP availability to cardiac myocytes and the potential initiation of apoptosis.

Within cardiac myocytes a signalling pathway of interest involves the role of ATP and the resulting activation of the AMPK pathway. AMPK is highly expressed within the myocardium (Stapleton *et al*. 1996), the activation of AMPK signalling is associated with protecting against cytotoxic effects in non-cancerous cells such as cardiac myocytes, inhibiting the release of EEF-2 (Terai *et al*. 2005). It was mentioned in the study by Kerkela *et al*. (2009) that in the presence of Sunitinib, ATP is unable to bind to AMPK, therefore AMPK is unable to transfer phosphate from ATP to the required substrates and therefore the energy conservation mechanism is not recruited, and energy depletion is exacerbated. Studies involving Sunitinib-direct inaction of AMPK signalling is limited, the review by Force *et al*. (2007) suggested the hypothetical inhibition of AMPK signalling. The study by Kerkela *et al*. (2009) demonstrated the potential for Sunitinib to inhibit AMPK signalling, whilst over-expression of a constitutively active mutant AMPK was able to ameliorate Sunitinib-induced cardiac myocyte cytotoxicity. If this is correct, the inactivation of AMPK could result in the release EEF-2, mTOR and ACC from inhibition and potentially contribute towards exacerbating ATP depletion as mentioned (McLeod and Proud 2002, Moore and Brophy 1994, Terai *et al*. 2005, Dennis *et al*. 2001). Moreover, the study by Laderoute e*t al*. (2010) demonstrated that Sunitinib competitively binds to AMPK, as potent as the AMPK-inhibitor Compound C. As reported, the resulting depletion of ATP would result in the activation of apoptosis and necrosis and cardiac myocyte loss, contractile and ventricular dysfunction (Fliss and Gattinger 1996, Webster *et al*. 1999, Doenst *et al*. 2013, Gupta *et al*. 2009, Weiss *et al*. 2005, Ingwall 1993, Bolling *et al*. 1991). This highlights the complexity and importance of investigating the role of AMPK signalling in both drug-induced cardiotoxicity and cancer cell death.

In contrast, Metformin hydrochloride, used to treat type-2 diabetes and insulin resistance, is associated with cardioprotective properties within ischaemic settings (Paiva *et al*. 2009, Paiva *et al*. 2010, Bhamra *et al*. 2008, and Barreto-Torres *et al*. 2012). The exact mechanism of Metformin-induced cardioprotection is still unknown, however Metformin is postulated to be involved in a number of anti-apoptotic activating pathways. Several suggested routes of cardioprotection by Metformin involves the inhibition of mTOR (Melnik and Schmitz 2014), the phosphorylation of the Akt signalling pathway (Bhamra *et al*. 2008), PPAR (Barreto-

Torres *et al*. 2012). Metformin's anti-hyperglycaemic ability is dependent on the activation of AMPK (Rena *et al*. 2013); the phosphorylation of AMPK was shown to be mediated by the increase in the cytosolic AMP concentration (Zhang *et al*. 2007). The stimulation of the adenosine receptor was shown to be an important mechanism in infarct size-limiting effects of ischaemic pre- and post-conditioning (Yellon and Downey 2003), whilst adenosine receptor stimulation was shown to activate Akt (Yang *et al*. 2004) and inhibit the opening of mPTP (Hausenloy *et al*. 2002). In particular the AMPK pathway is vital in response of cells to hypoxic conditions and ischaemia in the heart (Hardie and Hawley 2001, Frederich *et al*. 2005), therefore cardiac myocyte survival may be affected in events of hypoxia and ischaemia. Activation of AMPK protects against hypoxia-induced cardiac myocyte death via intracellular ATP conservation (Terai *et al*. 2005). It must be critically noted that the inhibition of AMPK signalling is a crucial pathway for tumour cell death, therefore re-designing Sunitinib to avoid AMPK inhibition is unavoidable as described by Kerkela *et al*. (2009) and a compromise must be drawn when applying extracellular stimuli leading to AMPK activation or when attempting to initiate a form of potential cardioprotection via AMPK activation to counter Sunitinib-induced heart damage. However, as discussed Metformin has anti-cancer properties via the activation of AMPK, therefore it is crucial to investigate the role of AMPK in both cardioprotection and cancer treatment. This further reinforces the importance of investigating the direct role of AMPK involvement, with the possibility of designing adjunctive therapy in order to maintain the activation of AMPK. Metformin has currently been trialled in studies involving patients undergoing Sunitinib treatment (Keizman *et al*. 2016, Hamieh *et al.* 2017), demonstrating a potential to improve overall survival in diabetic patients with metastatic renal cell carcinoma whilst also demonstrating an improvement in cardiovascular prognosis and reducing incidences of MI within patients (UKPDS 1998).

Metformin demonstrated a cardioprotective effect in ischaemia-reperfusion in rats via the activation of PPAR, in particular PPARα (Barreto-Torres *et al*. 2012, Oidor-Chan *et al*. 2016, Wayman *et al*. 2002). PPARα is highly expressed in the heart and plays a role in maintaining cardiac metabolic homeostasis (Yue *et al*. 2003). Moreover, it was highlighted by Oidor-Chan *et al*. (2016) that PPARα ligands were shown to reduce myocardial ischaemia-reperfusion injury in diabetic and non-

diabetic animal models, involving the anti-inflammatory mechanism of PI3K, the Akt and nitric oxide pathways (Dumitrescu *et al*. 2007, Yue *et al*. 2003, Wayman *et al*. 2002). From this, Metformin's ability to activate PPARα could result in the activation of fatty acid oxidation enzyme activity and fat metabolism, previously reported to be decreased following ischaemia-reperfusion (Yue *et al*. 2003). The activation of fatty acid oxidation enzyme by PPARα is suggested to improve the ability of the heart to use fatty acid as an energy source and maintain adequate energy production in the heart (Yue *et al*. 2003). Furthermore, activation of PPARα was shown to potentially attenuate the accumulation of neutrophils and proinflammatory cytokines, associated with the reperfusion cardiac injury (Yue *et al*. 2003).

Using this knowledge, we aim to investigate the potential for Metformin to demonstrate cardioprotective properties during Sunitinib administration in order to potentially attenuate Sunitinib-induced cardiotoxicity in Langendorff heart experiments. The haemodynamic measurements of LVDP, HR and CF will be measured to investigate heart function during adjunctive administration. Moreover, TTC staining will be carried out to measure infarct percentage following treatment.

3.2. Methodology

3.2.1. Chemicals

Metformin hydrochloride (500 mg, MW 165.62 mg/mM) was purchased from Sigma Aldrich. Metformin was dissolved in ultra-pure RO water; the stock solution was stored at -20 ^{o}C. Sunitinib Malate (25 mg, MW 532.56 mg/mM) was purchased from Sigma Aldrich. Sunitinib was dissolved DMSO; the stock solution was stored at -20 ^{o}C and care was taken to avoid exposure to light and UV radiation during use and storage. Remaining lab reagents were purchased from Fisher Scientific.

3.2.2. Isolated perfused heart preparation and Langendorff protocol.

SD rats (2-3 month old male, 345–375 g b/w, Charles River research laboratories, Margate, UK) were sacrificed and hearts were immediately mounted onto the langendorff system and retrogradely perfused with KH buffer as described in Section 2.3.1. Functional parameters of LVDP, HR and CF were measured and recorded at regular intervals 5 minutes for 55 minutes, followed by intervals of 15 minutes for 120 minutes. At the end of the experiment hearts were weighed and frozen at -20 ^{o}C. Frozen hearts were sliced into 2 mm thick transverse sections before being incubated in TTC solution (1 % in phosphate buffer) at 37 ^{o}C for 10–12 minutes and fixed in 10 % formalin for a minimum of 4 hours. Infarct area was traced onto acetate sheets and infarct percentage (%) was calculated. Computerised Imagetool^{TM} software was used to analyse the percentage of infarct tissue. Infarct size was normalised to the total area of each heart slice.

All hearts were allowed to stabilise for 20 minutes prior to being subjected to drug treatment for 155 minutes (Figure 11, Section 2.3.1.-2.3.2.). Hearts were randomly assigned to the following groups; (i) vehicle control hearts perfused with KH buffer and DMSO final concentration <0.1 %; (ii) hearts perfused with KH buffer and Metformin hydrochloride (50 µM) for 155 minutes; (iii) hearts perfused with KH buffer and Sunitinib malate (1 µM) for 155 minutes; (iv) hearts perfused with KH buffer and Metformin (50 µM) and Sunitinib (1 µM) for 155 minutes.

3.2.3. Statistical Analysis.

The data were expressed as mean ± SEM. Haemodynamics LVDP, HR and CF were assessed for the statistical difference ($p<0.05$) using ANOVA with LSD post hoc tests for each specific time point. The use of the two-way repeated measures ANOVA was used to assess the statistical significance ($p<0.05$) between time and time against treatment group for HR, LVDP and CF. The infarct size percentage data was tested for group significant differences ($p<0.05$) using one-way ANOVA with LSD post hoc tests.

3.3.1. Results.

Results from 24 successful experiments were included for the Langendorff study to assess the effect on HR (Figure 12a), LVDP (Figure 12b) and CF (Figure 12c) during Metformin co-administration with Sunitinib in SD rat hearts. Experiments were carried out for vehicle control, Metformin (50 µM), Sunitinib (1 µM) and the combination (co-treatment) of Metformin (50 µM) and Sunitinib (1 µM).

3.3.2. The effects of Sunitinib in the absence and presence of Metformin on haemodynamic parameters in SD rat hearts.

Figure 12a the effects of drug treatment on HR as a percentage of mean stabilisation value

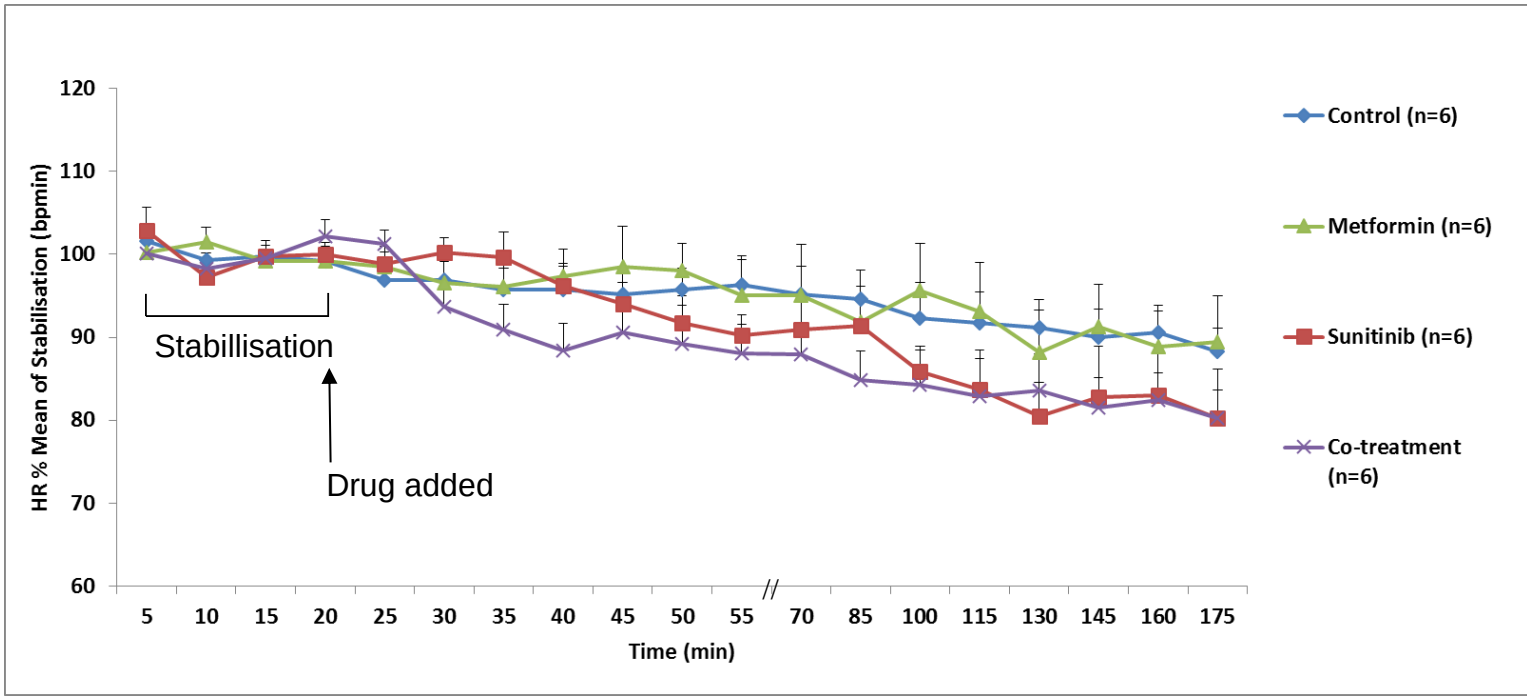

Figure 12a: The effects of drug treatment on HR as a percentage of mean stabilisation value: vehicle control (blue), Metformin (50 µM) (green), Sunitinib (1 µM) (red) and the combination of Metformin (50 µM) and Sunitinib (1 µM) (co-treatment, purple). Data is presented as mean ± SEM of 6 experiments (n=6).

From Figure 12a, Sunitinib treatment showed no significant changes to HR during the treatment period when compared to the vehicle control. Moreover, co-treatment showed no significant change to HR when compared to the Sunitinib treatment.

Single factor ANOVA, and post-hoc LSD during one-way ANOVA, determined no statistical significance between the co-treatment and 1 µM Sunitinib treatment group at selected time points ($p>0.05$) and no statistical significance between the 1 µM Sunitinib treatment group and the vehicle control group at selected time points ($p>0.05$). Single factor ANOVA and post-hoc LSD during one-way ANOVA determined no statistical significance for HR between treatment groups against vehicle control at selected time points ($p>0.05$).

Two-way repeated measures ANOVA, with post-hoc Tukey HSD during repeated measures ANOVA, demonstrated statistical significance for time on HR ($p<0.001$) for the Wilks' Lambda score but no statistical significance for time against treatment ($p>0.05$). As Mauchly's Test of Sphericity® was violated ($p<0.001$), from this Greenhouse-Geisser determined statistical significance for time on HR ($p<0.001$) and time against Treatment on HR ($p<0.05$). However, use of Tukey HSD post-hoc test for multiple comparisons determined no statistical significance between all treatment groups against the vehicle control on CF ($p>0.05$).

Figure 12b the effects of drug treatment on LVDP as a percentage of mean stabilisation value

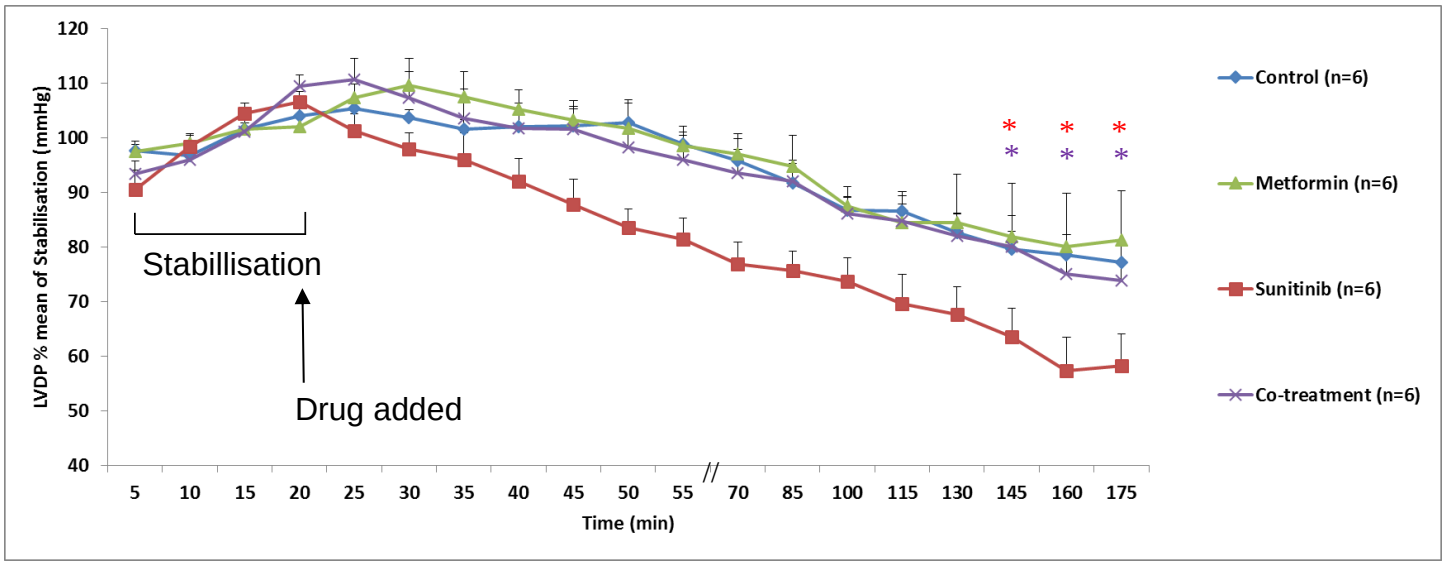

Figure 12b: The effects of drug treatment on LVDP as a percentage of mean stabilisation value: vehicle control (blue), Metformin (50 µM) (green), Sunitinib (1 µM) (red) and the combination of Metformin (50 µM) and Sunitinib (1 µM) (co-treatment, purple). Data is presented as mean ± SEM of 6 experiments (n=6). *Key statistical significance: *=p<0.05 1 µM Sunitinib vs vehicle control. *=p<0.05 1 µM Sunitinib vs Co-treatment.*

From Figure 12b, Sunitinib treatment showed a significant reduction in LVDP during the treatment period when compared to vehicle control. Co-treatment of Sunitinib with Metformin was demonstrated to attenuate this decrease in the mean LVDP when compared to time-matched Sunitinib treatment.

Single factor ANOVA and post-hoc LSD during one-way ANOVA determined statistical significance for LVDP for the 1 µM Sunitinib treatment group against the vehicle control group at selected time points 145, 160, 175 minutes ($p<0.05$. 145 minute; 64 ± 5 % vs. 80 ± 3 %, 165 minute; 57 ± 6 % vs. 79 ± 4 %, 175 minute; 58 ± 6 % vs. 77 ± 3 %).

Single factor ANOVA and post-hoc LSD during one-way ANOVA determined statistical significance for the 1 µM Sunitinib treatment group against the co-treatment group at selected time points 145, 160, 175 minutes ($p<0.05$, 145 minute;

64 ± 5 % vs. 80 ± 6 %, 160 minute; 57 ± 6 % vs. 75 ± 3 %, 175 minute; 58 ± 6% vs. 74 ± 3%).

Two-way repeated measures ANOVA, with post-hoc Tukey HSD during repeated measures ANOVA, determined statistical significance for time on LVDP ($p<0.001$) for the Wilks' Lambda score but no statistical significance for time against treatment on LVDP ($p>0.05$).

Moreover, Mauchly's Test of Sphericity® was violated ($p<0.001$), from this Greenhouse-Geisser determined statistical significance for time on LVDP ($p<0.001$) and significance for time against treatment on LVDP ($p<0.05$). Moreover, use of Tukey HSD post-hoc test for multiple comparisons determined no statistical significance between the Sunitinib treatment group against the vehicle control ($p>0.05$) or Sunitinib against the co-treatment group ($p>0.05$).

Figure 12c the effects of drug treatment on CF as a percentage of mean stabilisation value.

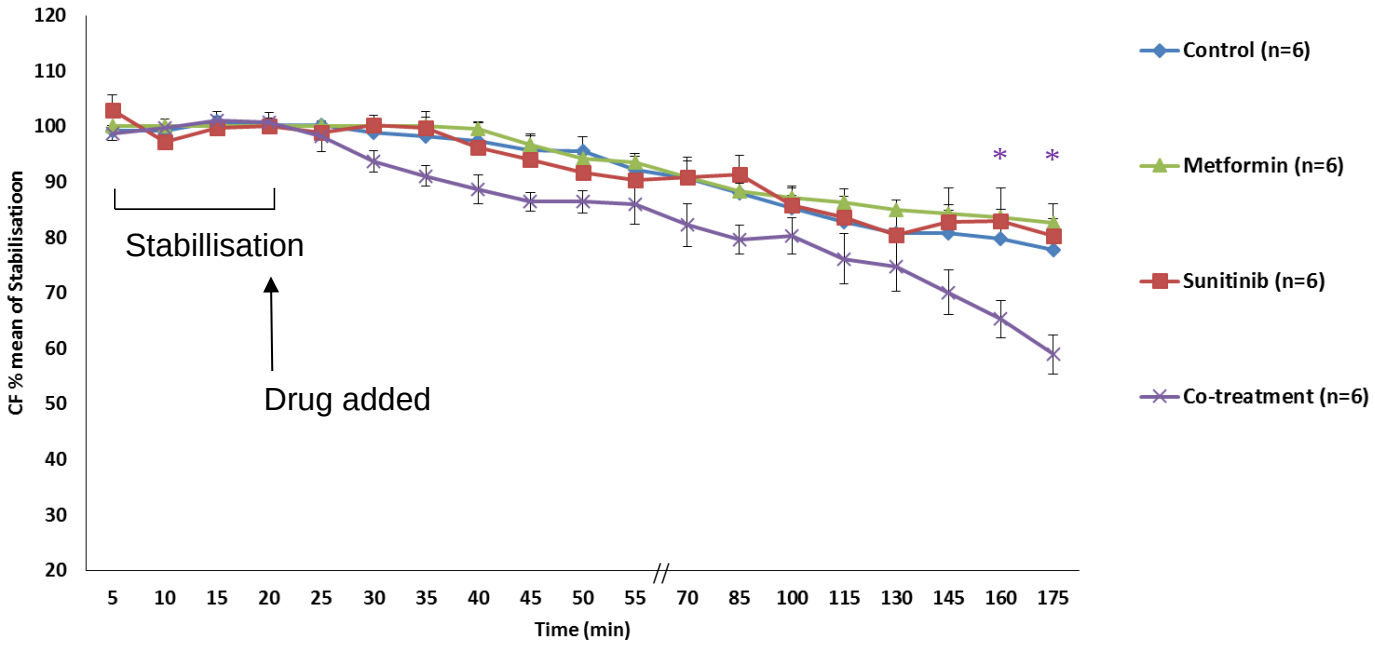

Figure 12c: The effects of drug treatment on CF as a percentage of mean stabilisation value: vehicle control (blue), Metformin (50 µM) (green), Sunitinib (1 µM) (red) and the combination of Metformin (50 µM) and Sunitinib (1 µM) (co-treatment, purple). Data is presented as mean ± SEM of 6 experiments (n=6). *Key statistical significance: *=p<0.05 1 µM Sunitinib vs. Co-treatment.*

CF was recorded by collecting the effluent for 1 minute at regular intervals; data was corrected for heart weight at the end of the Langendorff experiment and is presented as a percentage of mean stabilisation ± SEM in Figure 12c.

From Figure 12c, Sunitinib demonstrated no changes to CF when compared to vehicle control. Single factor ANOVA and post-hoc LSD during one-way ANOVA determined statistical significance for CF for the co-treatment group against the 1 µM Sunitinib treatment group at the 160 and 175 minute time points ($p<0.05$, 160 minutes; 65 ± 3 % vs. 83 ± 6 %, 175 minutes; 59 ± 3 % vs. 80 ± 6 %).

Furthermore, single factor ANOVA and post-hoc LSD during one-way ANOVA determined statistical significance for CF for the co-treatment group against the

vehicle control treatment group at the 160 and 175 minute time points ($p<0.05$, 160 minutes 65 ± 3 % vs. 80 ± 4 %, 175 minutes 59 ± 3 % vs. 78 ± 3 %, $p<0.05$).

Two-way repeated measures ANOVA, with post-hoc Tukey HSD during repeated measures ANOVA, determined statistical significance for time on CF ($p<0.001$) for the Wilks' Lambda score and statistical significance for time against treatment on CF ($p<0.05$).

Moreover, Mauchly's Test of Sphericity® was violated ($p<0.001$), from this Greenhouse-Geisser determined statistical significance for time on CF ($p<0.001$) and determined significance for time against treatment on CF ($p<0.001$). Moreover, use of Tukey HSD post-hoc test for multiple comparisons determined no statistical significance between the Sunitinib treatment group against the vehicle control ($p>0.05$) or against the co-treatment group ($p>0.05$). Tukey HSD post-hoc test demonstrated no significance for the co-treatment group against the vehicle control ($p>0.05$).

3.3.3. The effects of Sunitinib in the absence and presence of Metformin on infarct percentage in SD rat hearts.

Results from 24 successful experiments were included for the Langendorff study to assess the effect on infarct size percentage (Figure 13) during co-treatment of Metformin and Sunitinib in SD rat hearts (n=6). Experiments were carried out for vehicle control, Metformin concentration 50 µM, Sunitinib 1 µM and the combination (co-treatment) of Metformin (50 µM) and Sunitinib (1 µM).

Figure 13 the effects of drug treatment on infarct percentage

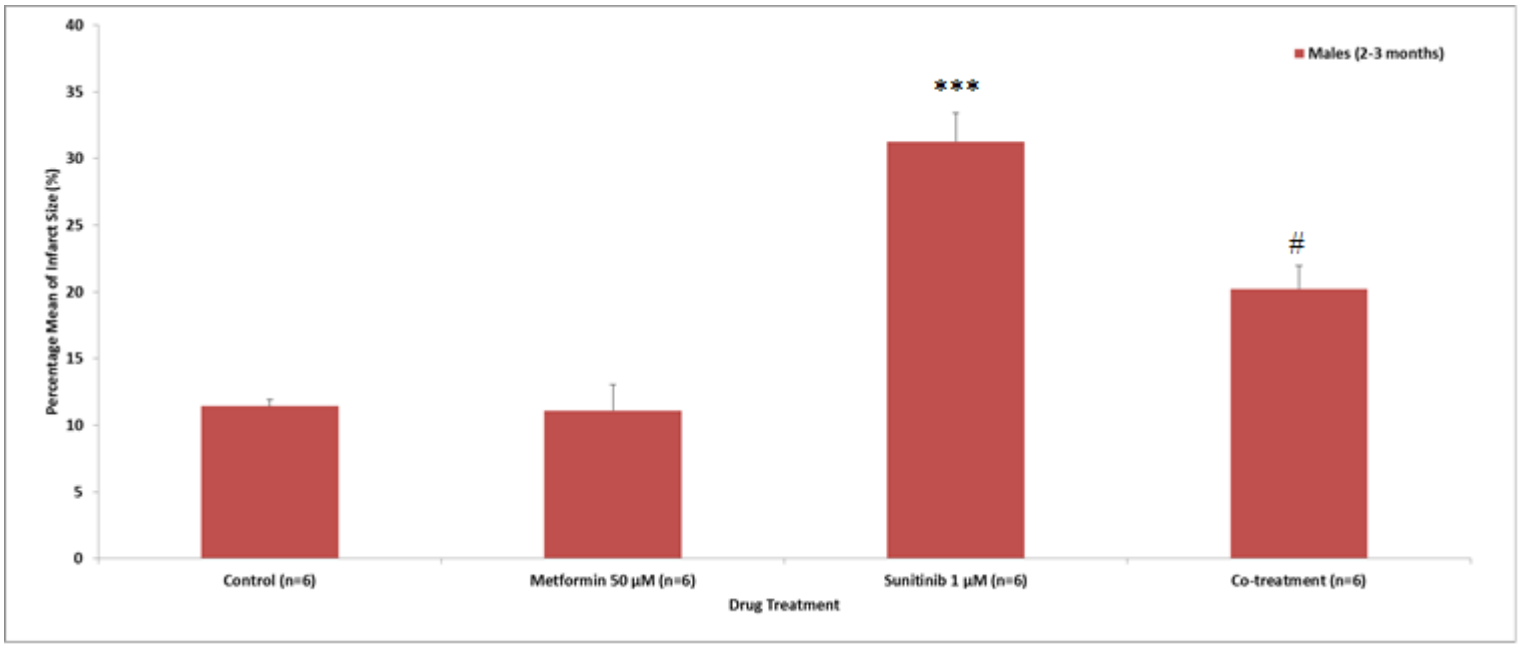

Figure 13: The effects of vehicle control, Metformin (50 µM), Sunitinib (1 µM) and the combination of Metformin (50 µM) and Sunitinib (1 µM) (Co-treatment) on infarct percentage (%). Data is presented as mean ± SEM of 6 experiments (n=6). *Key statistical significance: ***=p<0.001 1 µM Sunitinib vs. vehicle control. #=p<0.05 Co-treatment vs Sunitinib.*

Infarct size was determined as a percentage of the total area of the heart and presented in Figure 13.

From Figure 13, an increase in infarct percentage was demonstrated for the 1 µM Sunitinib treatment group in comparison to the vehicle control group ($p<0.001$, 31 ± 2 % vs. 11 ± 1 %). The co-treatment group demonstrated a significant reduction in infarct percentage compared to the 1 µM Sunitinib treatment group ($p<0.05$, 20 ± 2 % vs. 31 ± 2 %).

3.4. Discussion

We demonstrated that the multi-TKI Sunitinib (1 μM) was able to result in a significant decrease in LVDP at certain time points in SD rat hearts, compared to vehicle control using the Langendorff system (Figure 12b). It can be suggested that the resulting decrease in LVDP compared to vehicle control may be attributed to Sunitinib-induced energy impairment of cardiac function. The potential for Sunitinib inhibition of AMPK signalling and receptors PDGF and VEGF are said to result in and contribute towards energy impairment of cardiac function (Greineder *et al*. 2011, Gorini *et al*. (2018). In particular, VEGF was shown to be expressed in the heart and expression of VEGF is significantly increased in the infarcted myocardium during the early stage of MI (Li *et al*. 1996). Moreover, following MI VEGFR and PDGF are involved in the angiogenic occurs in order to carry out cardiac repair and scar tissue formation, with newly formed vessels shown in the infarcted myocardium (Zhao *et al*. 2010, Liu *et al*. 2014). The proposed effects are known to result from Sunitinib's targeting of PDGF, VEGF and AMPK in relation to Sunitinib's TKI ability in cancer treatment, targeting tumour proliferation and tumour angiogenesis via the inhibiting of energy required for cancer cell neoangiogenesis and growth which can arise from mutant TKs (Kerkela *et al*. 2009, Krause and Van Etten 2005, Chen *et al*. 2008, Folkman 2007, Jain *et al*. 2005).

The effects can also be attributed to Sunitinib's lack of selectivity and a possible increased risk of off-target toxicities by inhibiting additional kinases (Kerkela *et al*. 2009). Furthermore, the resulting inhibition of growth factor pathways by Sunitinib results in the impairment of cardiac energy homeostasis in cardiac myocytes (Chu *et al*. 2007, Chintalgattu *et al*. 2011). Sunitinib treatment at 1 μM was shown to result in energy deprivation and energy generation impairment in cardiac myocytes (Kerkela *et al*. 2009), the resulting effects of energy impairment in cardiac myocytes result in a decrease in heart function and therefore can contribute to a significant decrease in LV function as shown in Figure 12b (Jessen *et al*. 2010, Suematsu *et al*. 2003, Doenst *et al*. 2013, Ingwall 2008, Gorski *et al*. 2015, Harvey and Leinwand 2011, Corrado *et al*. 1997). In concordance with the proposed hypothesis of energy deprivation following Sunitinib administration, patients with metastatic and gastrointestinal stromal tumours, following Imatinib–resistance, were demonstrated

to improve LV dysfunction and symptoms following Sunitinib interruption, however episodic LV ejection fraction reductions were shown in some patients following Sunitinib administration restarting (Chu *et al*. 2007). The authors indicated that the contractile dysfunction may have occurred due to impaired ATP generation secondary to mitochondrial dysfunction and not from irreversible damage (Chu *et al*. 2007).

Results from Figure 13 demonstrated that Sunitinib at 1 µM significantly increased infarct percentage when compared to the vehicle control, highlighting the ability for Sunitinib to directly result in cardiac tissue damage using the Langendorff system, possibly acting via cardiac myocyte damage and activation of apoptosis. Sunitinib at 1 µM was selected in line with previous studies (Henderson *et al*. 2013) demonstrating the steady-state blood concentration of Sunitinib to be in the range of 0.1-1 µM (Goodman *et al*. 2007, Henderson *et al*., 2013). Results from Figure 13 are supported by a study demonstrating Sunitinib at 1 µM administration resulted in the significant increase in pro-apoptotic caspase-9 signalling (Chu *et al*. 2007). Moreover, mice fed with Sunitinib to produce blood concentrations comparable to patients, demonstrated abnormalities in cardiac myocytes including mitochondrial swelling (Chu *et al*. 2007). Furthermore, in cultured cardiac myocytes Sunitinib demonstrated the release of pro-apoptotic cytochrome-c into the cytosol (Chu *et al*. 2007), the release of cytochrome-c is known to activate the mitochondrial pathway for cellular death and apoptosis (Baines and Molkentin 2005). The same study by Chu *et al*. (2007) demonstrated Sunitinib to result in the activation of caspase-9 involved in the mitochondrial apoptotic pathway. Furthermore, in mice administered with Sunitinib to achieve a blood-plasma concentration similar to human patients resulted in the release of pro-apoptotic markers of chromatin condensation and distortion of mitochondrial cristae (Chu *et al*. 2007). Moreover, a recent study highlighted phenotypic changes indicative of cellular death in cardiac fibroblasts, including intracellular vacuoles, incubated with Sunitinib 1 µM (McBride *et al*. 2018). Using this, it can be suggested that Sunitinib is associated with the activation in cellular apoptosis of cardiac myocytes, resulting in the possible heart tissue damage and an increase in infarct percentage of the heart (Figure 13) during administration throughout the Langendorff study, however to confirm this, clinical biomarkers of cardiac myocyte damage and apoptosis would need to be investigated.

Studies by Sandhu *et al*. 2017 and Cooper *et al*. 2018 involving Sunitinib at 1 µM were able to demonstrate an increase in infarct percentage similar to our results obtained in Figure 13. Similar to our results obtained in Figure 12c, Sunitinib at 1 µM concentration did not demonstrate an alteration in CF when compared to vehicle control group (Sandhu *et al*. 2017). Sunitinib at 1 µM was demonstrated to result in an increase in infarct percentage similar to that obtained in Figure 13, when administered using the Langendorff technique (Sandhu *et al*. 2017, Cooper *et al*. 2018). Moreover, Sunitinib at 1 µM was demonstrated to result in a significant decrease in LVDP at certain time points during Langendorff perfusion using SD rat hearts (Sandhu *et al*. 2017). However in contrast to our results obtained in Figure 12a, Sunitinib was demonstrated to result in a significant decrease in HR (Sandhu *et al*. 2017), whilst our data demonstrated that Sunitinib did not result in an alteration of HR, the endpoint value of HR at 125 minutes obtained by Sandhu *et al*. (2017) is similar to the end point value of HR at 175 minutes shown in Figure 12a (83 ± 5 % vs. 80 ± 6 %). This highlights that, although the end-point value for Sunitinib from Figure 12a is similar to that from Sandhu *et al*. (2017), Sunitinib did result in a similar effect in our study but showed a greater degree of toxicity for the study by Sandhu *et al*. (2017) as shown by the significant decrease in HR compared to vehicle control. To explain this, although we tried to keep experimental conditions as similar as possible, the use of the Langendorff system requires attention to detail and two different Langendorff systems may result in a different set of results depending on the duration of time taken to hang the heart following cerebral dissection and the system itself. Moreover, the study by Sandhu *et al*. (2017), demonstrated an end-point value of (insert) for the vehicle control group, whereas the end-point value of HR in Figure 12a was 88 ± 3 %

Similar to the proposed mechanism of toxicity, the authors concluded that results obtained were in accordance to previous studies demonstrating Sunitinib's ability to induce cardiotoxicity via apoptosis and reports of LV dysfunction and HR in patients (Chu *et al*. 2007, Henderson *et al*. 2013). The study by Sandhu *et al*. (2017) further demonstrated that Sunitinib resulted in the increase of PKCα in heart tissue. PKCα is demonstrated to be activated during cellular apoptosis (Shimizu *et al*. 1998), this can be used to suggest that Sunitinib demonstrated an activation of apoptosis and

was able to result in an increase in infarction percentage of the heart tissue as shown in Figure 13.

Interestingly it was demonstrated from Figure 12c, co-administration of Metformin (50 µM) with Sunitinib (1 µM) resulted in a significant decrease in CF when compared to vehicle control at time points 160 and 175 minutes ($p<0.05$). This could suggest that the co-treatment of Metformin and Sunitinib resulted in a potential vasoconstrictive effect when administrated together. A possible explanation of this could be due to Metformin-associated and prolonged AMPK activation-associated with lactic acidosis (Vecchio and Protti 2011, Cerda-Kohler *et al*. 2018, Corremans *et al*. 2018). However, Metformin-induced lactic acidosis remains unclear among existing literature (Baradari *et al.* 2011, Misbin 2004). It is worth noting that the significant difference was shown only at the 160 and 175 minute time points and not during previous time points, highlighting that the effects are time-dependent and could be prevented with intervention or drug-discontinuation (Cezur *et al*. 2009, Yavari *et al*. 2016, Silvestre *et al*. 2007, Ashall and Dawes 2008).

As we discussed in Section 3.1., Sunitinib can potentially target VEGFRs on the surface of cardiac myocytes, thereby resulting in a pro-apoptotic response on non-cancerous cells. Results obtained in Figure 13 can be suggested to stem from the proposed activity, as the binding to and resulting apoptosis of cardiac myocytes would result in myocardial ischaemia, in particular the inhibition of the VEGF receptor by Sunitinib was shown to be associated with causing hypertension and myocardial ischaemia (Orphanos e*t al*. 2009). Neo-angiogenesis is critical for metastasis and the formation of vessels for tumour growth but also for growth of cardiac myocytes and the angiogenic response to MI, VEGF is considered to play a vital role in the process, moreover VEGF inhibitors such as Sunitinib has become an established treatment for tumour types (Lankhorst *et al*. 2015, Ellis and Hicklin *et al*. 2008, Kamba *a*nd McDonald 2007). However, as the heart is highly dependent on energy, it is more susceptible to damage that can result from an altered blood supply, the cardiovascular system is said to exhibit a higher sensitivity to Sunitinib damage when compared to other organs (Lankhorst *et al*. 2015). To support the proposed binding to VEGFRs, toxicity via Sunitinib administration was indicated to occur in the kidneys with glomerular and thrombotic microangiopathy was said to

be frequently observed due to disruption of the VEGF signalling pathway (Eremina *et al*. 2008, Vigneau *et al*. 2014, Maynard *et al*. 2003). The resulting action by Sunitinib would interfere with cellular signal transduction, cell cycle regulation metabolism and transcription, all contributing to an increased risk of cardiac events in cancer patients, with coronary microvessels implicated as the primary target of toxicity (Chintalgattu *et al*. 2013). In accordance with this, the study by Lankhorst *et al*. (2015) demonstrated that the severity of hypertension and renal injury are dose-dependent, it was highlighted that using a low dose of Sunitinib, equivalent to 50 mg/kg in patients, the concentration of the active metabolite N-desethyl in Sunitinib was higher than that observed in patients, indicating an increased metabolism of the compound in rats (Yu *et al*. 2015, Speed *et al*. 2012). Glomerular capillaries were also shown to be obliterated when using a high dose of Sunitinib; glomerular endotheliosis is considered a hallmark in angiogenesis inhibition-induced renal injury (Eremina *et al*. 2008).

As discussed, Metformin is associated with cardioprotective properties during ischaemia-reperfusion damage (Paiva *et* al. 2009, Paiva *et* al. 2010, Bhamra *et* al. 2008). Metformin has been further associated with anti-neoplastic actions that has led to studies investigating the molecular actions (Evans *et al*. 2005, Giovannucci *et al*. 2010, Ben Sahra *et al*. 2008, Zakikhani *et al*. 2006, Andrzejewski *et al*. 2014). For this reason, we favoured the use of Metformin over other protective agents as there is potential for Metformin to be used as an adjunctive form of cancer treatment with Sunitinib. We investigated to determine if Metformin can exhibit a potential similar cardioprotective effect during Sunitinib co-administration within a normoxic study Langendorff perfused rat hearts (Figures 12a–12c, 13). We demonstrated that Metformin when co-administered at 50 µM was able to significantly reduce infarct percentage in SD rat hearts when compared to Sunitinib treatment alone in the Langendorff experiment (Figure 13). It was also demonstrated that the administration of Metformin in co-treatment with Sunitinib was able to attenuate Sunitinib's induced significant decrease in LVDP as shown in Figure 12b.

As proposed previously, the hypothesis that Sunitinib inactivates or down regulates certain signalling pathways such as AMPK and results in energy deprivation, it can be suggested from results in Figures 12b and 13 that Metformin co-administration

was able to prevent this. As discussed, the potential for Sunitinib to induce energy deprivation and ATP depletion resulted in the significant decrease in LVDP in Figure 12b, the attenuation in LVDP decrease following Metformin co-administration may have resulted from Metformin's proposed ability to activate AMPK signalling during events of ischaemic stress in order to counter the reduction in ATP, to restore energy levels (Paiva *et* al. 2009, Paiva *et* al. 2010, Bhamra *et* al. 2008).

In support of our data obtained in Figures 12b and 13, similarities can be drawn to existing data. Paiva *et al*. (2010) demonstrated that Metformin (50 µM) administration was able to significantly reduce ischaemia-reperfusion-induced infarct percentage, suggesting that Metformin has cardioprotective properties. The concentration of 50 µM for Metformin was said to produce a higher peak plasma concentration within humans (Bhamra *et* al. 2008), however our data in Figure 13 demonstrated that this concentration did not result in a significant increase in drug-induced infarct percentage when compared to vehicle control.

Moreover, Wang *et al*. (2017) demonstrated that Metformin (100 µM) was able to reduce infarct size percentage when compared to the ischaemia-reperfusion control via an AMPK-activation manner whilst Xu *et al*. (2014) demonstrated that Metformin protected against systolic overload-induced HR. Wang *et al*. (2017) further demonstrated that Metformin (100 µM) administration 15 minutes prior to ischaemia was able to attenuate the ischaemia-induced LVDP decrease improving LV function when compared to normoxic control. Using this knowledge, it can be suggested that results obtained in Figures 12b and 13 were as a result of Metformin attenuating Sunitinib-induced cardiotoxicity via AMPK activation, reversing the significant decrease in LVDP and the increase in infarct percentage. Moreover, it can be suggested that in order for Metformin to initiate a potential cardioprotective response via AMPK activation, Metformin administration will need to be continuously perfused during the period of cardiac damage, either via ischaemia or drug-induced, as demonstrated by the study by Hasinoff *et al*. (2008) of which cultured cardiac myocytes were incubated with Metformin prior to removal of Metformin and incubation with Sunitinib. The study showed that Metformin treatment, and removal, prior to Sunitinib administration did not demonstrate cardioprotective properties.

Hasinoff *et al*. (2008) demonstrated that Sunitinib was able to result in a dose-dependent lactate-dehydrogenase (LDH) release in cardiac myocytes, at concentrations similar to peak plasma concentrations in a therapeutic single-day dose (Deeks and Keating 2006, Faivre *et al*. 2006). Sunitinib further inhibited the enzyme activity of AMPK and RSK1 at IC_{50} concentrations 0.32 µM and 0.36 µM, both concentrations were said to be within the therapeutic plasma concentration range and therefore may potentially contribute to cardiotoxicity induced by Sunitinib (Hasinoff *et al*. 2008, Deeks and Keating 2006, Faivre *et al*. 2006, Chu *et al*. 2007, Telli *et al*. 2008). However, the authors concluded that because the kinase inhibition assays used, measured 50 µM ATP which was said to be much less than the mM intracellular ATP concentration, the IC_{50} values would increase due to competition with ATP (Hasinoff *et al*. 2008).

As AMPK is a known sensor of cellular energy status and activation controls ATP, it is worth mentioning that cardiac tissue, unlike other tissue, contains a reserve of ATP, therefore impaired ATP production could be a factor in Sunitinib-induced cardiotoxicity (Hasinoff *et al*. 2008, Hardie 2004). Hasinoff *et al*. (2008) attempted to demonstrate if inhibition of AMPK by Sunitinib could result in cardiotoxicity by measuring the effect on intracellular ATP levels in cardiac myocytes, however following the 4-hour treatment with Sunitinib the authors concluded that they were unable to demonstrate if Sunitinib did result in myocyte death by reducing ATP levels (Hasinoff *et al*. 2008). The dosages of 5 and 10 µM were able to decrease pACCα levels in cardiac myocytes, whilst doses of 0.5, 1, 5, and 10 µM demonstrated significance against non-treated vehicle control when investigating caspase–3/7 activity (Hasinoff *et al*. 2008). A Sunitinib concentration of 0.1 µM is said to be approximately equal to that of a therapeutic plasma concentration (Hasinoff *et al*. 2008, Deeks and Keating 2006, Faivre *et al*. 2006), as concluded by the authors, the previously mentioned concentrations were agreed to be much greater than the therapeutic Sunitinib plasma levels, with 5 µM said to be approximately 25-fold greater, it is difficult to conclude if therapeutic doses could significantly inhibit AMPK to determine Sunitinib-induced cardiotoxicity (Hasinoff *et al*. 2008, Deeks and Keating 2006, Faivre *et al*. 2006). However, it should be mentioned that the study by Hasinoff *et al*. (2008) only subjected cardiac myocytes to 2 hours of treatment with Sunitinib, whereas patients undergoing Sunitinib

treatment would be administered daily for an extended period of time, therefore continuous inhibition of AMPK by Sunitinib could occur as a result of long-term treatment and could potentially have more subtle effects in inducing cardiotoxicity (Hasinoff *et al*. 2008). Moreover, the cellular levels of Sunitinib in daily administered doses are unclear and could be potentially greater than the stated doses (Hasinoff *et al*. 2008).

In contrast to the measured caspase-3 levels, levels of the pro-apoptotic Bax were shown to be not significant following Sunitinib treatment, suggesting that the involvement of this pathway was not present in the induction of apoptosis, whilst Sunitinib was found to have no significant effect on oxidative stress on cardiac myocytes (Hasinoff *et al*. 2008). Furthermore, the pre-treatment of cardiac myocytes with AMPK activators Metformin or Phenformin failed to protect against Sunitinib-induced damage (Hasinoff *et al*. 2008, Bertrand *et al*. 2006). This adds to the continuing debate amongst existing literature in regards to the exact route of Sunitinib-induced cardiotoxicity.

It has been mentioned that during an event of ischaemia, activation of AMPK within the myocardium is considered to enhance glucose uptake, utilise anaerobic glucose and produce sufficient ATP required for cardiac myocyte function (Paiva *et al*. 2010, Dyck and Lopashuk 2006). The resulting action prevents cardiac myocyte death and allow the heart to readjust from anaerobic glucose metabolism to aerobic oxidative metabolism of glucose (Paiva *et al*. 2010, Dyck and Lopashuk 2006).

Using this knowledge, it can be suggested that Metformin's potential mode of cardioprotection is associated with the activation of the AMPK signalling pathway (Gundewar *et al*. 2009). It has been said that upon activation, AMPK results in the inhibition of protein translation, protein synthesis and fatty acid synthesis, conserving ATP consumption, whilst activating AMP generating pathways via increasing fatty acid oxidation and glycolysis to counter the loss of ATP (Towler and Hardie 2007). Studies have shown AMPK to protect cardiac myocytes from anoxic damage, associated with anti-apoptotic activity (Igata *et al*. 2005, Capano *et al*. 2003, Shibata *et al*. 2005, Russell *et al*. 2004, Arad *et al*. 2007). The proposed activation of AMPK signalling by Metformin following Sunitinib co-administration can be reinforced with a study demonstrating AMPK signalling to be restored following

the gene transferring of active AMPK in a study involving Sunitinib-administration in cardiac myocytes (Kerkela *et al*. 2009). Moreover, Metformin was able to attenuate pressure-overload-induced cardiac hypertrophy within non-diabetic mice via the activation of AMPK, preventing cardiac myocyte loss (Fu *et al*. 2011). Metformin was further demonstrated to inhibit cardiac myocyte apoptosis and improving cardiac function via AMPK activation (Gundewar *et al*. 2009, Sasaki *et al*. 2009). Using this knowledge, it can be suggested that Metformin was able to attenuate Sunitinib-induced LVDP decrease and infarct percentage increase via AMPK activation to counter the loss of ATP, energy re-balance and salvage cardiac myocyte cell death following Sunitinib-administration.

Moreover, aside from AMPK several other non-direct mechanisms and signalling pathways may be contributing towards the associated effects shown in our results. The potential cardioprotective effects by Metformin in Figures 12a-13 can be hypothesised to stem from an AMPK-independent effect. It can be suggested Metformin is acting to inhibit Sunitinib-induced mPTP formation, Sunitinib has been said to result in mPTP formation following activation of apoptosis during administration (Gorini *et al*. 2018, Kerkela *et al*. 2009, Chu *et al*. 2007). mPTP formation within cardiac myocytes and the myocardium is in correlation with an increase in infarct percentage and a decrease in heart function (Bhamra *et* al. 2008), as shown in Figures 12b and 13. To support this, studies have demonstrated the activation of the reperfusion injury salvation kinase (RISK) pathway kinase Akt following Metformin administration during ischaemia, the subsequent activation of RISK/Akt acts to inhibit mPTP and result in a decrease in infarct percentage (Hausenloy *et* al. 2007, Bhamra *et* al. 2008, Whittingham *et al*. 2013, Driver *et al*. 2018, Sridharan *et al*. 2016). However to conclude this, the direct investigation of AMPK, RISK and/or Akt signalling following Metformin co-administration with Sunitinib would need to be investigated.

From Figure 12a, use of the two-way repeated measures ANOVA, with post-hoc Tukey HSD during repeated measures ANOVA, demonstrated that there was significant difference for time on HR ($p<0.001$), moreover statistical significance was demonstrated for time against treatment on HR ($p<0.05$). From this, it can be observed in Figure 12a that there is a gradual decrease in HR for the Sunitinib and

co-treatment groups against the vehicle control, however when comparing each treatment group against individual time points no significance was shown for time on HR and time against treatment at individual time points using the repeated measures ANOVA with Tukey HSD ($p>0.05$). Moreover, to confirm this the single factor ANOVA used for Figure 12a also demonstrated that there was no significance between each treatment group at individual time points ($p>0.05$). From Figure 12a, it is shown that both time and time against treatment is having a significant effect on HR as shown by a gradual decrease in HR following all treatments, this is somewhat expected as the Langendorff technique itself is a system that does not prevent the isolated perfused heart form dying, it is expected that there will be a gradual decrease in haemodynamic parameters as the technique itself is described as a dying procedure (Bell *et al*. 2011). As mentioned by Bell e*t al.* (2011), a 10 % deterioration of contractile strength was shown using the Langendorff system. However, when investigating the perfusion of a drug such as Sunitinib it was shown that the observed changes were more significant, when compared to vehicle control such as shown in Figure 12b.

From Figure 12b, use of the two-way repeated measures ANOVA multiple factor demonstrated significant difference for time on LVDP, as well as time against treatment on LVDP ($p<0.001$). However in contrast, no significance was shown for the Sunitinib treatment group against the vehicle control, as well as for the Sunitinib treatment group against the co-treatment group using Tukey HSD ($p>0.05$). As the use of the repeated measures ANOVA determined significance for the effects of time and time against treatment on LVDP, this demonstrated that time and time against treatment had an effect on LVDP in Figure 12b. However, the use of the post-hoc Tukey HSD demonstrated no significance for time and time against treatment for Sunitinib against vehicle control overall and Sunitinib against co-treatment overall ($p>0.05$). However, when investigating individual time points, the use of the single-factor ANOVA determined significance for Sunitinib against vehicle control and Sunitinib against co-treatment ($p<0.05$).

From Figure 12c, significance was determined for time on CF and time against treatment on CF ($p<0.001$). However, no significance was determined for the Sunitinib treatment against vehicle control, as well as the Sunitinib treatment group

against co-treatment ($p>0.05$). Moreover, no significance was demonstrated for the co-administration treatment group against the vehicle control group for individual time points ($p>0.05$). In contrast, when investigating individual time points, it was determined using the single-factor ANOVA that significance was shown for the co-treatment group against the vehicle control at the 160 and 175 minute time points ($p<0.05$).

In contrast to our results obtained in Figures 12b and 13, the Carotid Atherosclerosis: Metformin for Insulin Resistance (CAMERA), and the Glycometabolic Intervention as Adjunct Primary Percutaneous Coronary Intervention in ST–Segment Elevation Myocardial Infarction (GIPS) III randomised trial studies failed to demonstrate protective properties of Metformin (Preiss *et al*. 2014, Lexis *et al*. 2014). No significance among LVEF or markers of cardiovascular disease (carotid intima-media thickness) were found when administering 1g/day Metformin for 4 months vs placebo/control in patients who previously underwent primary percutaneous coronary intervention for ST-segment elevation myocardial infarction or patients taking statins who were not diagnosed with type 2 diabetes but did have coronary heart disease (Preiss *et al*. 2014, Lexis *et al*. 2014). However, as highlighted by many authors, the use of 1g/day of Metformin may not be enough to demonstrate an impact on endpoint of LVEF or any potential cardioprotective effect, whilst the use of carotid intima-media thickness as a potential marker for cardiovascular disease is in debate (Pauriah *et al*. 2008). However, the UK Prospective Diabetes Study from 1998 and Holman *et al*. (2008) were able to demonstrate that Metformin was able to significantly decrease the risk of diabetes-related cardiovascular end points and all-cause death when compared to conventional therapies after accounting for blood glucose levels.

The study by Kravchuk *et al*. (2011) demonstrated that Metformin did not significantly affect the HR, LVDP and CF amongst control and type-2 diabetes mellitus rats, pre-administered with Metformin 200mg/kg, during ischaemia-reperfusion, suggesting that Metformin does not have a cardioprotective effect during pre-treatment and does not result in myocardial pre-conditioning. However, similar to the study by Hasinoff *et al*. (2008) of who carried out Metformin pre-treatment prior to Sunitinib administration, Metformin was administered prior to a

form of ischaemia and therefore was not able to override ATP depletion that was caused by the ischaemia itself. The authors further mentioned that drug-washout may have occurred at the time of ischaemia-reperfusion (Kravchuk *et al*. 2011), it would be worth seeing if this effect can be reversed if Metformin was Langendorff-perfused during ischaemia-reperfusion. Despite this study, many studies involving Metformin administration within the Langendorff model are modelled around preventing ischaemic injury at the time of, or following, reperfusion injury therefore it is difficult to draw a direct comparison with our data. We demonstrated that co-administration of Metformin with Sunitinib in Figure 12c significantly lowered CF at the 160 and 175 minute time points when compared to vehicle control, however Metformin administration alone did not significantly lower CF when compared to vehicle control. Moreover, the co-treatment of Metformin and Sunitinib was shown to attenuate Sunitinib-induced decrease in LVDP (Figure 12b) and not have an effect on HR (Figure 12a) when compared to vehicle control. Following this, it is difficult to explain the reduction in CF at these time points and further work would be required to investigate the reduction in CF. If we choose to explore AMPK signalling it is worth noting that persistent activation of AMPK during reperfusion results in glycolysis being favoured ahead of pyruvate oxidation, increases acidosis and reduces cardiac efficiency during the period of recovery, adding one possible explanation for the reduction in CF at time points 160 and 175 minutes in Figure 12c (Paiva *et al*. 2010). Moreover, the coadministration of Metformin and Sunitinib could potentially be resulting in a vasoconstrictive effect and thusly lowering CF at these time points shown in Figure 12c. Sunitinib has previously been shown to result in vasoconstriction and hypertension, affecting the endothelin without an increase in oxidative stress (Kappers *et al*. 2011). Using this information, it could be suggested that although Metformin is producing a cardioprotective effect in combination with Sunitinib, as shown by the restoration of LVDP and infarct percentage, Sunitinib is able to override some of the potential effects of Metformin and thusly can result in a vasoconstrictive effect. Interestingly, from Figure 12c Sunitinib administration alone did result in a decrease in CF when compared to vehicle control. The study by Sandhu *et al*. (2017) demonstrated that Sunitinib-induced changes to LVDP and HR were able to be overcome with the addition of the protective agent IB-MECA, however the addition of IB-MECA was not able to attenuate Sunitinib-induced decrease in CF compared to Sunitinib treatment alone.

Using this, it can be suggested that Sunitinib may be able to override the protective effects of IB-MECA and carry out a vasoconstrictive effect, as suggested for the possible decrease in CF resulting from the coadministration of Sunitinib and Metformin from Figure 12c.

However, the resulting decrease at the 160 and 175 minute time-points of CF for the coadministration of Sunitinib and Metformin may be due to certain properties of Metformin. As mentioned previously reports of Metformin-induced lactic acidosis exists, the statement by Page *et al*. (2016) further indicated Metformin as a possible contributor to exacerbating underlying myocardial dysfunction, moreover the author's highlighted Metformin as a '*major*' category for '*Magnitude of HF Induction or Precipitation*'. Page *et al*. (2016) further stated that the possible reason for this was due to Metformin-associated increase in anaerobic metabolism and elevated lactic acidosis, with an immediate or delayed onset of effects. Although information presented by Page *et al.* (2016) is contraindicated by information presented in the statement, the information presented highlights the importance of monitoring Metformin treatment, particularly in the context of HR, in multiple parameters of haemodynamics as well as infarct percentage. As well as this, Metformin was demonstrated to have inotropic effects, the study by Teng *et al*. (2015) demonstrated that Metformin was able to degrade phospholamban located in the sarcoplasmic reticulum membrane of mouse neonatal cardiac myocytes via an autophagic-associated response, phospholamban acts to regulate Ca^{2+} uptake in the sarcoplasmic reticulum in cardiac myocytes. This suggests that Metformin may have certain inotropic effects within the heart which could account for certain changes on haemodynamic parameters. However, more research would be required to investigate this effect within our experimental model as we reported no changes to HR and LVDP during Metformin treatment alone. The decrease in CF following cotreatment of Metformin and Sunitinib could be resulting from myocardial edema as Metformin is also reported to cause edema in certain parts of the body, mainly legs and feet. However, the effects of edema on the heart are reported with Thiazolidinedione treatment (Kermani and Garg 2003), with very few sources indicating Metformin-induced pulmonary edema and has not been reported by either the National Institute for Health and Care Excellence (National Institute for Health and Care Excellence 2019) or by manufacturers (Bristol-Myers Squibb 2018).

Taking this into consideration, Metformin combination therapy has previously been demonstrated in studies. The study by Olsson *et al*. (2000) demonstrated the increase in cardiovascular mortality in patients with T2DM taking Sulfonylurea and Metformin in comparison to Sulfonylurea monotherapy. The author's concluded that this combination of therapy cannot exclude for the possibility of increasing cardiovascular mortality (Olsson *et al*. 2000). However, it is worth mentioning the study by Olsson *et al*. (2000) carried out a comparison of 741 patients undergoing Sulfonylurea therapy alone and 169 patients undergoing Sulfonylurea and Metformin combination therapy, whilst the authors mentioned that the increase in mortality was secondary to a more aggressive form of diabetes present in the combination therapy group. However, our results indicated that Metformin administration alone throughout the Langendorff protocol following 20 minutes stabilisation, did not significantly reduce HR, LVDP or CF when compared to the normoxic control, whilst in coadministration with Sunitinib significantly attenuated the increase in infarct percentage caused by Sunitinib treatment alone and attenuated Sunitinib-induced decrease in LVDP.

3.5. Conclusion.

To conclude, we observed an increase in infarct percentage in SD rat hearts when administered with the multi-TKI Sunitinib malate at the relevant dose of 1 µM, using the Langendorff technique. Moreover, Sunitinib was demonstrated to result in a significant decrease in LVDP when compared to vehicle control. Both HR and CF were demonstrated to not be altered during Sunitinib administration using the Langendorff technique compared to the vehicle control. The decrease in LVDP and increase in infarct percentage were both attenuated when co-administered with the type-2 diabetes agent Metformin hydrochloride at 50 µM concentration. Moreover, Metformin administration alone was demonstrated to not alter haemodynamics and infarct percentage compared to vehicle control, however the coadministration of Metformin and Sunitinib resulted in a significant decrease in CF at the 160 and 175 minute time points, when compared to the vehicle control. The proposed mechanism of Sunitinib-induced cardiotoxicity was discussed in Section 3.4., whilst the proposed mechanism of Metformin-induced cardioprotection was touched upon in the same chapter. The direct role of Metformin-induced cardioprotection in Sunitinib-induced cardiotoxicity will explored and discussed further in Chapters 4 and 5, whilst the proposed mechanism of AMPK-signalling involvement will be further explored in Chapter 4.

Chapter Four: Inhibition of the human equilibrative nucleoside transporter prevents Metformin-induced cardioprotection in Sunitinib-induced cardiotoxicity, the role of AMPK activation.

Disclaimer: Results presented in Sections 4.3.1 and 4.3.2. were previously presented in Sections 3.3.1., 3.3.2 and 3.3.3. Sections 4.3.1. and 4.3.2. builds on this study and incorporates the use of NBTI and the combination of Metformin, Sunitinib and NBTI.

Data and contents in this chapter were published / presented as following:

Conferences

- British Pharmacological Society Annual Conference (2018) London, England.
- 2nd MCRN meeting at Aston University, (2018), Birmingham, England.
- British Society of Cardiovascular Research Autumn Meeting (2017), Oxford, England.
- Safety Pharmacology Society and National Centre for the Replacement, Refinement and Reduction of Animals in Research: The use of human tissues for safety assessment, Regional meeting (2017), Coventry, England.

Abstract and poster presentation

- The Role of AMPK Signalling During Metformin Co-administration with Sunitinib, British Pharmacological Society Annual Conference (2018), London, England.
- The Role of AMPK Signalling During Metformin Co-administration with Sunitinib, 2nd MCRN meeting at Aston University, (2018), Birmingham, England.
- *The Assessment of the Cardioprotective Properties of Metformin during Sunitinib-Induced Cytotoxicity,* British Society of Cardiovascular Research Autumn Conference (2017), Oxford, England.
- *The Cardioprotective Properties of Metformin during Sunitinib-Induced Cytotoxicity*, Safety Pharmacology Society and National Centre for the

Replacement, Refinement and Reduction of Animals in Research: The use of human tissues for safety assessment, Regional meeting (2017) Coventry, England.

4.1. Introduction

As discussed in Chapter 1, cardiotoxicity is described as a broad range of adverse effects on heart function that can ultimately lead to HF and death. Cardiac muscle tissue relies heavily on aerobic metabolism as a source of ATP due to the tissue being highly energetic (Mellor e*t al*. 2011). The use of chemotherapeutic agents for the treatment of cancer has been associated with an increased risk of cardiovascular complications. During cancer treatment, when the cancer is said to be incurable the improvement in overall survival via treatment with a potentially cardiotoxic agent will outweigh the associated safety risk (Mellor *et al*. 2010). Moreover, if the drug treatment can be used as an adjunctive form, the balance between risk and benefit will need to be examined carefully. To date, the TKI Sunitinib is administered, despite reports of adverse cardiac events, due to the drug being highly effective within settings where treatment is limited especially during oncology drug development where there is an issue in achieving anticancer efficiency is great, the risk of cardiotoxicity is considered on balance (Mellor *et al*. 2010). New concerns exist regarding targeted drugs where cardiac effects are either unknown, not anticipated or detected in pre-clinical studies such as that with TKI Sunitinib (Orphanos *et al*. 2009, Henderson *et al*. 2013).

We were able to demonstrate that Sunitinib when administered at 1µM significantly increased infarct percentage compared to the vehicle control, in Sections 3.1.-3.5., and the combination with Metformin at 50 µM was shown to attenuate this increase. From this, we aimed to investigate the signalling pathway associated with Metformin-induced cardioprotective properties in combination with Sunitinib. AMPK has previously been proposed as a protein of interest during Sunitinib-induced cardiotoxicity as well as Metformin-induced cardioprotection (Laderoute *et* al. 2010, Force *et al*. 2007, Paiva *et al*. 2009, Paiva *et al*. 2010, Bhamra *et al*. 2008).

AMPK, both protein and messenger RNA (mRNA), is highly expressed in cardiac and skeletal tissue compared to liver in both rat studies and human studies (Verhoeven *et al*. 1995, Gao *et al*. 1995, Beri *et al*. 1994, Aguan *et al*. 1994). However, as mentioned by Kudo *et al*. (1996), it is unlikely that cardiac AMPK acts to regulate lipid biosynthesis, due to fatty acid and cholesterol biosynthesis being not important pathways in the heart, moreover the role of AMPK in cardiac tissue

may be to regulate fatty acid oxidation. Within the liver, AMPK phosphorylates and inactivates ACC, however ACC is also expressed in the heart and has a role in fatty acid oxidation and malonyl-CoA production for CPT-1 inhibition, CPT1 acts to uptake fatty acid in the mitochondria (Hardie 1992, Thampy 1989, Bianchi *et al*. 1990, McGarry *et al*. 1978). Fatty acids provide 60-70 % of the energy requirement for the heart, during reperfusion following ischaemia, fatty acid oxidation was shown to recover and provide 90–100% of the energy requirement for the heart (Neely and Morgan 1974, Liedtke *et al*. 1988, Lopaschuk *et al*. 1990).

The study by Kudo *et al*. (1995) demonstrated that the decrease in ACC activity and malonyl-CoA levels following ischaemia-reperfusion result in an accelerated rate of fatty acid oxidation, whilst the decrease in ACC activity resulted in an increase in AMPK activity. Following this, AMPK activation was shown to be present following ischaemic stress using post-mitochondrial extracts from rat heart tissue (Kudo *et al*. 1996). The resulting activation of AMPK phosphorylates and inactivates ACC (Kudo *et al*. 1996), in accordance with previous studies highlighting ACC's role in regulating cardiac fatty acid oxidation. Using this, it can be suggested that AMPK acts to regulate myocardial fatty acid oxidation (Kudo *et al*. 1996).

Within cardiac tissue, AMPK acts to protect cardiac myocytes following ischaemic stress via intracellular ATP conservation, a potential form of cardioprotection (Paiva *et al*. 2010, Hardie and Hawley 2001, Frederich *et al*. 2005, Terai *et al*. 2005). During ischemia, cardiac AMPK is active following reduced oxygen levels, an increase in 5'AMP levels and nutrient supply with oxidative phosphorylation resulting in the reduction of ATP production (Lopaschuk 2008, Kudo *et al.* 1995, Kudo *et al*. 1996, Weekes *et al*. 1994). Mitochondria are known to play an important role in events such as cardiac ischaemia-reperfusion, it was mentioned by Murphy and Steenbergen (2011) that defects in the electron transport chain contributes towards energy deprivation of the myocardium and result in increased mitochondrial generation of ROS and extensive oxidative damage. Therefore, preventing mitochondrial stress, mPTP formation and preserving mitochondria are vital for cardiac myocyte survival. Continuing from this, acute Metformin treatment was shown to improve myocardial energy metabolism during sustained ischaemic insult via upregulating NO production as a cardioprotective effect (Kawabata and Ishikawa 2003), the same effects were demonstrated among diabetic murine hearts (Calvert

et al. 2008) and delay the opening of mitochondrial transition pores in cardiac myocytes, a PI3K-dependent manner (Bhamra *et al*. 2008).

Continuing from results shown in Sections 3.1.-3.5., the role of AMPK signalling in Metformin-induced cardioprotection was a key focus. As mentioned in Section 1.14., the use of NBTI was demonstrated to inhibit Metformin-induced AMPK activation is settings of ischaemia-reperfusion (Paiva *et al*. 2010). NBTI is described as a high affinity binding and tight-binding specific inhibitor of ENT1, whilst also previously used to indicate ENT1 protein expression on the plasma membrane of cardiac myocytes (Cass *et al*. 1974, Chaudary *et al*. 2002, Rose *et al*. 2010). Within cardiovascular physiology, the purine nucleoside adenosine signalling molecule acts to reduce stress and regulate energy use within the cell (Mubagwa and Fleming 2001, Rose *et al*. 2009). Adenosine is required for ATP synthesis, nucleic acid formation whilst further initiating a cardioprotective response during an imbalance in oxygen demand and availability (Rose *et al*. 2010, Headrick e*t al*. 2003, Mubagwa and Fleming 2001, Pelleg *et al*. 2002). Cardioprotection involving purines in the cardiovascular system are widely investigated and accepted to involve murine nucleoside, adenosine, adenosine receptors and their associated signalling pathways, metabolic enzymes and purine nucleoside transporters (Rose *et al*. 2010). However, few studies exist demonstrating the nucleoside transporters in cardiovascular physiology (Rose *et al*. 2010).

Although the use of Metformin as a cardioprotective agent in co-treatment is nothing new, few studies exist involving the co-treatment of Metformin and Sunitinib, moreover Metformin's potential ability to demonstrate cardioprotective properties during Sunitinib-induced cardiotoxicity is limited. The study by Hasinoff *et al*. (2008) demonstrated that cardiac myocytes were not protected from Sunitinib-induced cell death following Metformin pre-treatment. The authors aimed to demonstrate if activation of AMPK was able to prevent Sunitinib-induced myocyte damage, the authors further demonstrated that the clinically approved Doxorubicin-cardioprotective agent Dexrazoxane was also unable to prevent Sunitinib-induced myocyte damage (Hasinoff *et al*. 2008). However, it was suggested that Sunitinib did not induce oxidative damage within cultured cardiac myocytes (Hasinoff *et al*. 2008). It must be noted that the study involved the pre-treatment of cardiac myocytes with Metformin; this was followed by Metformin removal and replaced with

Sunitinib. From this, it is difficult to conclude if AMPK was still active following incubation with Sunitinib.

From this, we previously demonstrated in Chapter 3, that Sunitinib at 1 µM was able to significantly increase infarct percentage of SD rat hearts during Langendorff perfusion. We aim to follow this up by investigating the effects of Sunitinib-induced cardiotoxicity directly in isolated cardiac myocytes. Using the information presented in Section 3.1.-3.5., as well as the aforementioned studies, it can be hypothesised that Sunitinib has a direct cardiotoxic role on cardiac myocytes, potentially resulting in the activation of pro-apoptotic pathways or acting on the mitochondria directly. Moreover, as presented using the Langendorff system involving SD rat hearts, Metformin was able to attenuate the increase in infarct percentage when co-administered together with Sunitinib. We aim to demonstrate the similar effect on isolated cardiac myocytes.

Using this knowledge, we aim to investigate the role of AMPK signalling in Metformin-induced cardioprotection and Sunitinib-induced cardiotoxicity. We have previously reported in Section 3.1.–3.5., that Sunitinib demonstrated cardiotoxic properties, resulting in an increase in infarct percentage of rat hearts during Langendorff perfusion. It was suggested that this may have resulted in Sunitinib activating apoptotic pathways as well as acting to limit ATP and energy to the myocardium, possibly via inhibiting AMPK signalling. We were able to attenuate this via the co-administration of Metformin.

As discussed in Section 3.4., we used existing studies and literature to suggest that this may have resulted from Metformin's ability to activate AMPK signalling in co-administration with Sunitinib. We aim to demonstrate the ability for Metformin to activate AMPK signalling during co-administration with Sunitinib in the Langendorff system, in doing so aim to inhibit the proposed activity of AMPK via the use of the ENT1 signalling antagonist NBTI, as demonstrated in studies by Paiva *et al*. (2010) and Aymerich *et al*. (2006). We aim to demonstrate this using Western blot analysis involving isolated rat myocardial tissue following Langendorff perfusion.

The information presented contributes to the debate surrounding the use of Metformin in cardioprotection in existing studies; nevertheless we aim to use this information to propose the potential for Metformin to activate AMPK signalling

during the course of Sunitinib administration using the Langendorff study. In doing so, we aim to potentially inhibit the route of Metformin-induced AMPK activation by using NBTI to potentially inhibit the recruitment of extracellular adenosine needed for AMPK activation. We further aim to demonstrate the direct phosphorylation of AMPK during all mentioned treatments, by investigating the phosphorylation of AMPK at the Thr^{172} residue using the Western blot analysis.

4.2. Methodology.

4.2.1. Chemicals.

Metformin hydrochloride (500 mg, MW 165.62 mg/mM) was purchased from Sigma Aldrich and dissolved in ultra-pure RO water. The Metformin stock solution was stored at -20^{o}C.

Sunitinib malate (25 mg, MW 532.56 mg/mM) was purchased from Sigma Aldrich and dissolved in DMSO. The Sunitinib stock solution was stored at -20^{o}C and care was taken to avoid exposure to light and UV radiation.

S-(4-Nitrobenzyl)-6-thioinosine (NBTI, 100 mg, MW 419.41 mg/mM) was purchased from Sigma Aldrich and was dissolved in DMSO. The NBTI stock solution was stored at -20^{o}C.

AMPKα, Phospho-AMPKα (pAMPK, Thr^{172}) and GAPDH antibodies were purchased Cell Signalling Technology® (New England BioLabs), and stored at -20 ^{o}C.

Remaining reagents were standard laboratory reagents from Sigma Aldrich and Fisher Scientific.

4.2.2. Isolated perfused heart preparation.

As described in Section 2.3.1., SD rats (2-3-month-old male, 345–375 g b/w, Charles River research laboratories, Margate, UK) were sacrificed and hearts were immediately mounted onto the langendorff system and retrogradely perused with KH buffer. Functional parameters of LVDP, HR and CF were measured and recorded at regular intervals 5 minutes for 55 minutes, followed by intervals of 15 minutes for 120 minutes. At the end of the experiment hearts were randomly selected to be used for (i) TTC staining or (ii) Western blotting. Hearts selected for (i) TTC staining were weighed and frozen at -20^{o}C. Frozen hearts were sliced into 2 mm thick transverse sections before being incubated in TTC solution (1 % in phosphate buffer) at 37^{o}C for 10–12 minutes and fixed in 10 % formalin for a minimum of 4 hours. Total area and infarct area were traced onto acetate sheets and infarct percentage (%) was calculated. Computerised Imagetool^{TM} software was used to analyse the percentage of infarct tissue. Infarct size was normalised to the total area of each heart slice. Hearts selected for (ii) Western blot analysis had the left atrium removed and snap frozen in liquid nitrogen. Samples were stored in -80 ^{o}C.

4.2.3. Langendorff protocol.

As described in Section 2.3.1., all hearts were allowed to stabilise for 20 minutes prior to being subjected to drug treatment for 155 minutes (Figure 11). Hearts were randomly assigned to the following group; (i) vehicle control hearts perfused with KH buffer and DMSO final concentration <0.1%; (ii) hearts perfused with KH buffer and Metformin hydrochloride (50 µM) for 155 minutes; (iii) hearts perfused with KH buffer and Sunitinib malate (1 µM) for 155 minutes; (iv) hearts perfused with KH buffer and Metformin (50 µM) and Sunitinib (1 µM) for 155 minutes, (v) hearts perfused with KH buffer and NBTI (1 µM) for 155 minutes, (vi) hearts perfused with KH buffer and Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM) for 155 minutes.

4.2.4. Isolated cardiac myocyte protocol.

SD rat left ventricular cardiac myocytes were isolated by conventional enzymatic dissociation (Maddock *et al*. 2002). Hearts were immediately isolated and mounted onto a modified langendorff apparatus. Hearts were perfused with modified KH bicarbonate buffer for 5 minutes followed by 8 minutes of perfusion with collagenase buffer, as described in Section 2.6.1.

After perfusion with collagenase the heart was removed, and the atrium was cut away before undergoing manual dissociation with collagenase buffer as described in Section 2.6.1. Samples were centrifuged and supernatant was removed before remaining pallet of viable tissue was resuspended in restoration buffer and calcium concentration was restored. Viability was determined to be >70 %.

4.2.5. Trypan Blue staining for percentage live cell population of cardiac myocytes.

Sunitinib 1 µM, Metformin 50 µM and/or NBTI 1 µM was added to each appropriate well at concentrations with vehicle control. Cardiac myocyte cell suspension was added to each appropriate well, making each well final volume 1 ml. Cells were incubated for 4 hours in the normoxic incubator (37^{o}C, 95 % O_2, 5 % CO_2) before undergoing trypan blue (1:1 dilution, 2x concentration) staining for live cell population counts.

4.2.6. Western blot detection of phosphorylated-AMPK (Thr^{172}), AMPKα and GAPDH.

As described in Section 2.4., a total of approximately 45-50 mg of frozen left ventricular tissue from the left atrium was lysed in lysis buffer before undergoing protein calculation for 60 µg, using Pierce™ BCA protein assay kit (ThermoFisher Scientific). Protein was diluted with sample buffer as described in Section 2.4., before loading on Mini-PROTEAN TGX stain-free gel (BioRad, USA) and transferred onto membrane electrophoresis with Tran-Blot Turbo (BioRad, USA). Immunoblots are analysed using primary antibodies phosphorylated AMPK (p-AMPK, Thr^{172}), total AMPKα and GAPDH. Computerised Imagetool™ software was used to quantify bands.

4.2.7. Statistical Analysis.

All data was expressed as mean ± SEM. Haemodynamics LVDP, HR and CF were assessed for the statistical difference ($p<0.05$) using one-way ANOVA with LSD post hoc tests for each time point value. The use of the two-way repeated measures ANOVA was used to assess the statistical significance ($p<0.05$) between time and time against treatment group for HR, LVDP and CF with post hoc Tukey HSD. The infarct size percentage data was tested for group significant differences ($p<0.05$) using one-way ANOVA with LSD post hoc tests. Cardiac myocytes for live-cell population were assessed for the statistical difference ($p<0.05$) using one-way ANOVA with LSD post hoc tests. Western blot analysis for p-AMPK, AMPKα and GAPDH were assessed for the statistical difference ($p<0.05$) using one-way ANOVA with LSD post hoc tests.

4.3. Results.

Continuing from Section 3.3.1., results from 36 successful SD rat heart experiments were included in the Langendorff study to assess the effect on HR (Figure 14a), LVDP (Figure 14b) and CF (Figure 14c) during Metformin (50 μM) co-administration with Sunitinib (1 μM), and Metformin co-administration with Sunitinib and AMPK inhibitor NBTI (1 μM).

Experiments were carried out for vehicle control, Metformin 50 μM, Sunitinib 1 μM, the combination (co-treatment) of Metformin (50 μM) and Sunitinib (1 μM), NBTI 1 μM and the combination (co-treatment and NBTI) of Metformin (50 μM), Sunitinib (1 μM) and NBTI (1 μM). As mentioned, data expressed for vehicle control, Metformin, Sunitinib and co-treatment were previously demonstrated in Section 3.1.-3.5.

4.3.1. The effects of Sunitinib in the absence and presence of Metformin and NBTI on haemodynamic parameters in SD rat hearts.

Figure 14a the effects of drug treatment on HR as a percentage of mean stabilisation

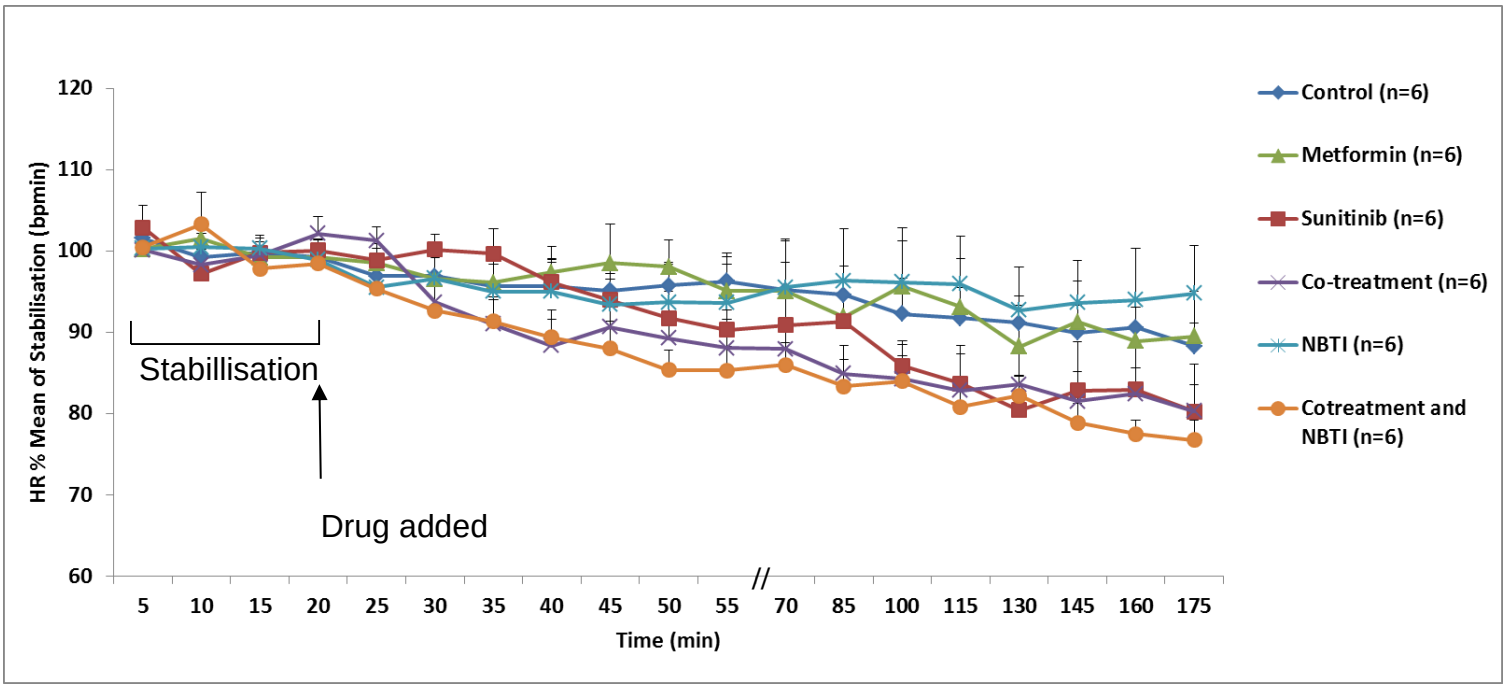

Figure 14a: The effects of drug treatment on HR as a percentage of mean stabilisation: vehicle control (blue), Metformin (50 µM) (green), Sunitinib (1 µM) (red), the combination of Metformin (50 µM) and Sunitinib (1 µM) (co-treatment, purple), NBTI (1 µM) (turquoise), and the combination of Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM) (co-treatment and NBTI, orange). Data is presented as mean ± SEM of 6 experiments (n=6).

From Figure 14a, Sunitinib treatment demonstrated no statistical significant changes to HR during the treatment period when compared to the vehicle control, whilst co-treatment showed no significant change to HR when compared to the Sunitinib treatment, as previously highlighted in Figure 12a. The combination of Metformin, Sunitinib and NBTI (co-treatment and NBTI) demonstrated no significant change compared to the combination of Metformin and Sunitinib (co-treatment). The use of NBTI alone demonstrated no significant change to HR when compared to vehicle control.

Single factor ANOVA and post-hoc LSD during one-way ANOVA determined no statistical significance between the co-treatment group and co-treatment and NBTI treatment group at selected time points ($p>0.05$).

Two-way repeated measures ANOVA, with post-hoc Tukey HSD during repeated measures ANOVA, determined statistical significance for time on HR ($p<0.001$) for the but no statistical significance for time against Treatment on HR ($p>0.05$) for the Wilks' Lambda score. Moreover, Mauchly's Test of Sphericity® was violated ($p<0.001$), from this Greenhouse-Geisser determined statistical significance for time on HR ($p<0.001$) and time against treatment on HR ($p<0.05$). However, use of Tukey HSD post-hoc test for multiple comparisons determined no statistical significance between all treatment groups against the vehicle control on CF ($p>0.05$), moreover Tukey HSD determined no statistical significance for Sunitinib against co-treatment ($p>0.05$), and no significance for co-treatment against co-treatment and NBTI ($p>0.05$).

Figure 14b the effects of drug treatment on LVDP as a percentage of mean stabilisation

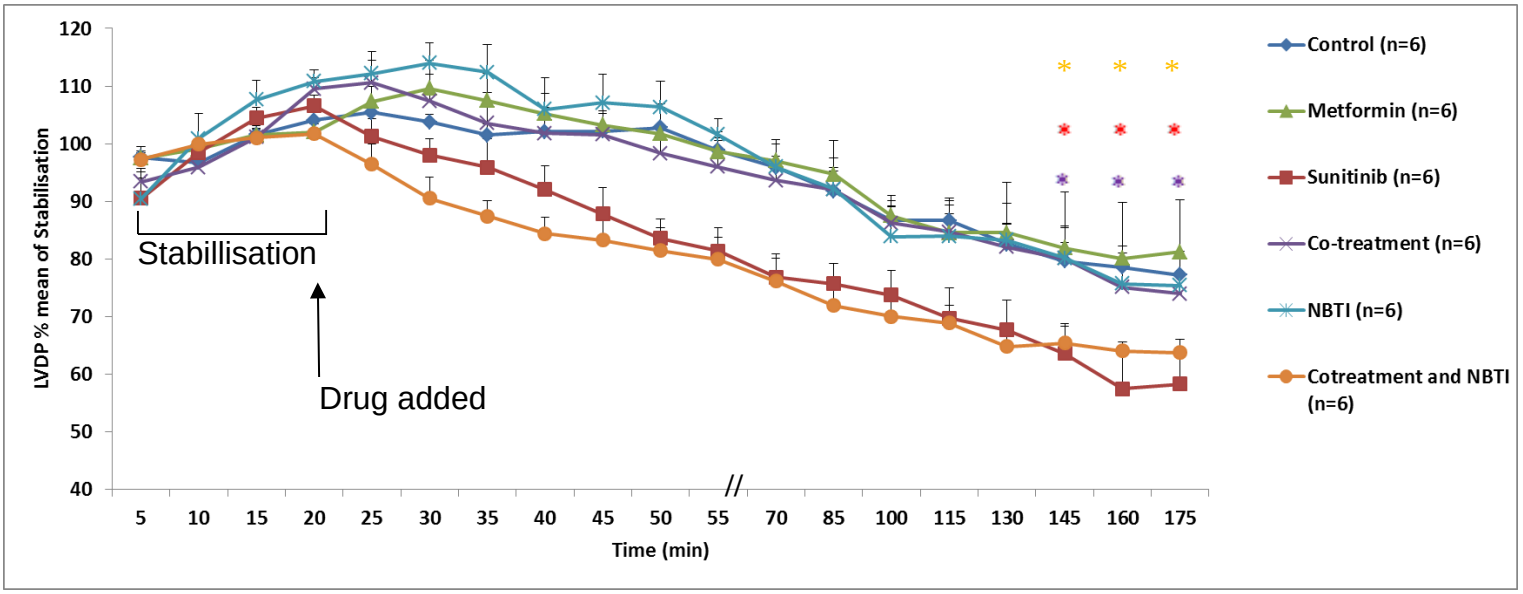

Figure 14b: The effects of drug treatment on LVDP as a percentage of mean stabilisation: vehicle control (blue), Metformin (50 µM**)** (green), Sunitinib (1 µM) (red), the combination of Metformin (50 µM) and Sunitinib (1 µM) (co-treatment, purple), NBTI (1 µM) (turquoise), and the combination of Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM) (co-treatment and NBTI, orange). Data is presented as mean ± SEM of 6 experiments (n=6). *Key statistical significance: *=p<0.05 1µM Sunitinib vs vehicle control. *=p<0.05 1µM Sunitinib vs Co-treatment. *=p<0.05 Co-treatment vs Co-treatment and NBTI.*

From Figure 14b, as previously highlighted in Figure 12b Sunitinib treatment resulted in a significant reduction in LVDP during the treatment period when compared to vehicle control, the co-treatment of Sunitinib with Metformin was demonstrated to attenuate this decrease in LVDP when compared to time-matched Sunitinib treatment. From Figure 14b, the co-treatment and NBTI group demonstrated an overall decrease in LVDP compared to the co-treatment group, similar to that of Sunitinib treatment. NBTI alone demonstrated no significant changes in LVDP compared to vehicle control.

Single factor ANOVA and post-hoc LSD during one-way ANOVA determined statistical significance for the co-treatment group against the co-treatment and NBTI group at selected time points 145, 160, 175 minutes ($p<0.05$, 145 minute; 80 ± 6 % vs. 65 ± 3 %, 160 minute; 75 ± 3 % vs. 64 ± 2 %, 175 minute; 74 ± 3 % vs. 64 ± 2 %).

Two-way repeated measures ANOVA, with post-hoc Tukey HSD during repeated measures ANOVA, determined statistical significance for time on LVDP ($p<0.001$) but no statistical significance for time against treatment on LVDP ($p>0.05$), for the Wilks' Lambda score. Moreover, Mauchly's Test of Sphericity® was violated ($p<0.001$), from this Greenhouse-Geisser determined statistical significance for time on LVDP ($p<0.001$) and significance for time against treatment on LVDP ($p<0.05$). Moreover, use of Tukey HSD post-hoc test for multiple comparisons determined statistical significance for the co-treatment and NBTI group against vehicle control ($p<0.05$), and for the co-treatment and NBTI group against the co-treatment group ($p<0.05$).

Figure 14c the effects of drug treatment on CF as a percentage of mean stabilisation

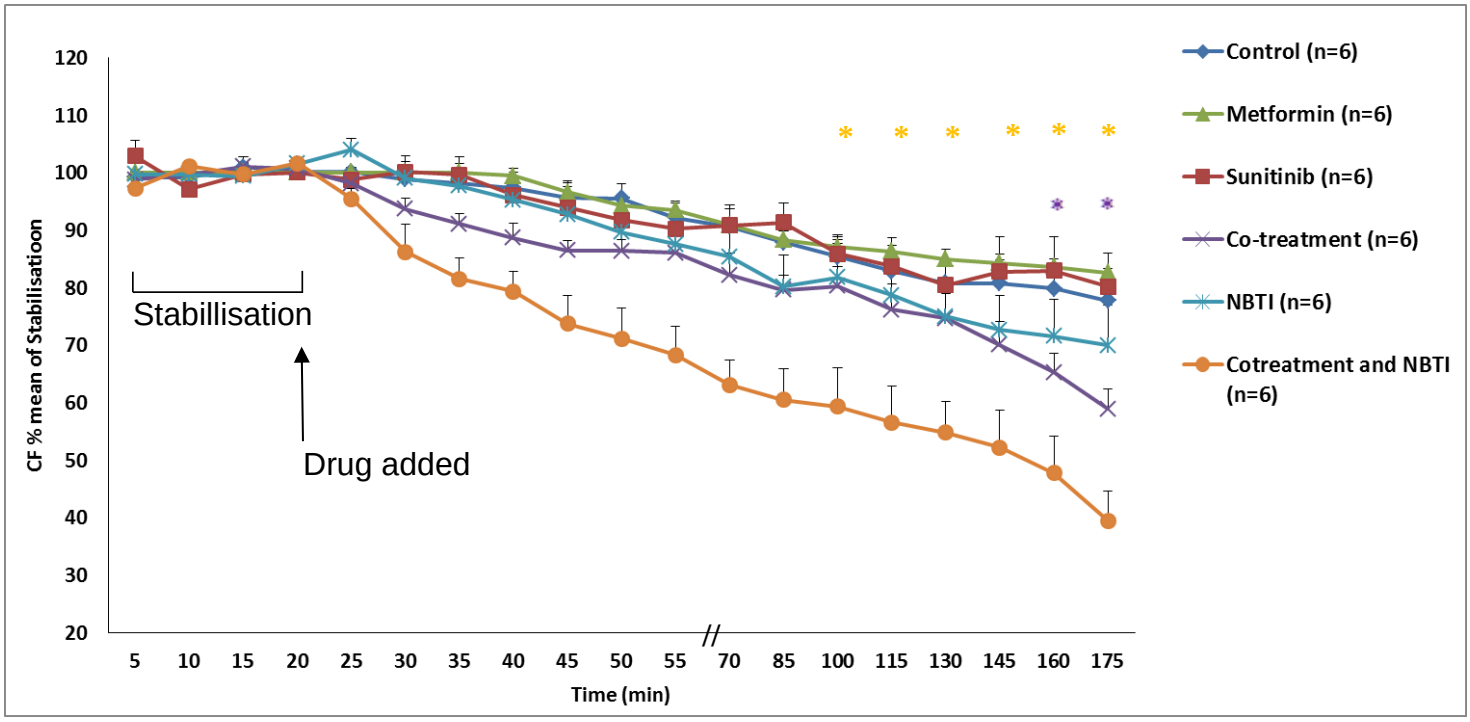

Figure 14c: The effects of drug treatment on CF as a percentage of mean stabilisation: vehicle control (blue), Metformin (50 µM) (green), Sunitinib (1 µM) (red), the combination of Metformin (50 µM) and Sunitinib (1 µM) (co-treatment, purple), NBTI (1 µM) (turquoise), and the combination of Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM) (co-treatment and NBTI, orange). Data is presented as mean ± SEM of 6 experiments (n=6). *Key statistical significance: *=p<0.05 1µM Sunitinib vs Co-treatment. *=p<0.05 Co-treatment vs Co-treatment and NBTI.*

CF was recorded by collecting the effluent for 1 minute at regular intervals; data was corrected for heart weight and is presented as a percentage of mean stabilisation ± SEM in Figure 14c.

It was demonstrated in Figure 14c that the co-treatment and NBTI group resulted in an overall decrease in CF compared to the co-treatment group alone. Single factor ANOVA and post-hoc LSD during one-way ANOVA determined statistical significance for CF for the co-treatment and NBTI group against the co-treatment group at selected time points 100, 115, 130, 145, 160 and 175 minutes ($p<0.05$,

100 minute; 59 ± 7 % vs. 80 ± 3 %, 115 minute; 57 ± 6 % vs. 76 ± 5 %, 130 minute; 55 ± 5 % vs. 75 ± 4 %, 145 minute; 52 ± 7 % vs. 70 ± 4 %, 160 minute; 48 ± 6 % vs. 65 ± 3 %, 175 minute; 39 ± 5 % vs. 59 ± 3 %).

Two-way repeated measures ANOVA, with post-hoc Tukey HSD during repeated measures ANOVA, determined statistical significance for time on CF ($p<0.001$) and statistical significance for time against treatment on CF ($p<0.05$) for the Wilks' Lambda score. Moreover, Mauchly's Test of Sphericity® was violated ($p<0.001$), from this Greenhouse-Geisser determined statistical significance for time on CF ($p<0.001$) and determined significance for time against treatment on CF ($p<0.001$). Moreover, use of Tukey HSD post-hoc test for multiple comparisons determined statistical significance for the co-treatment and NBTI group against the co-treatment group ($p<0.05$).

4.3.2. The effects of Sunitinib in the absence and presence of Metformin and NBTI on infarct percentage in SD rat hearts.

Results from 36 successful experiments were included for the Langendorff study to assess the effect on infarct size percentage (Figure 15) during co-treatment of Metformin, Sunitinib and NBTI in SD rat hearts (n=6). Experiments were carried out for vehicle control, Metformin concentration 50 µM, Sunitinib 1 µM, the combination (co-treatment) of Metformin (50 µM) and Sunitinib (1 µM), NBTI 1 µM and the combination (co-treatment and NBTI) of Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM).

Figure 15 the effects of drug treatment on infarct percentage

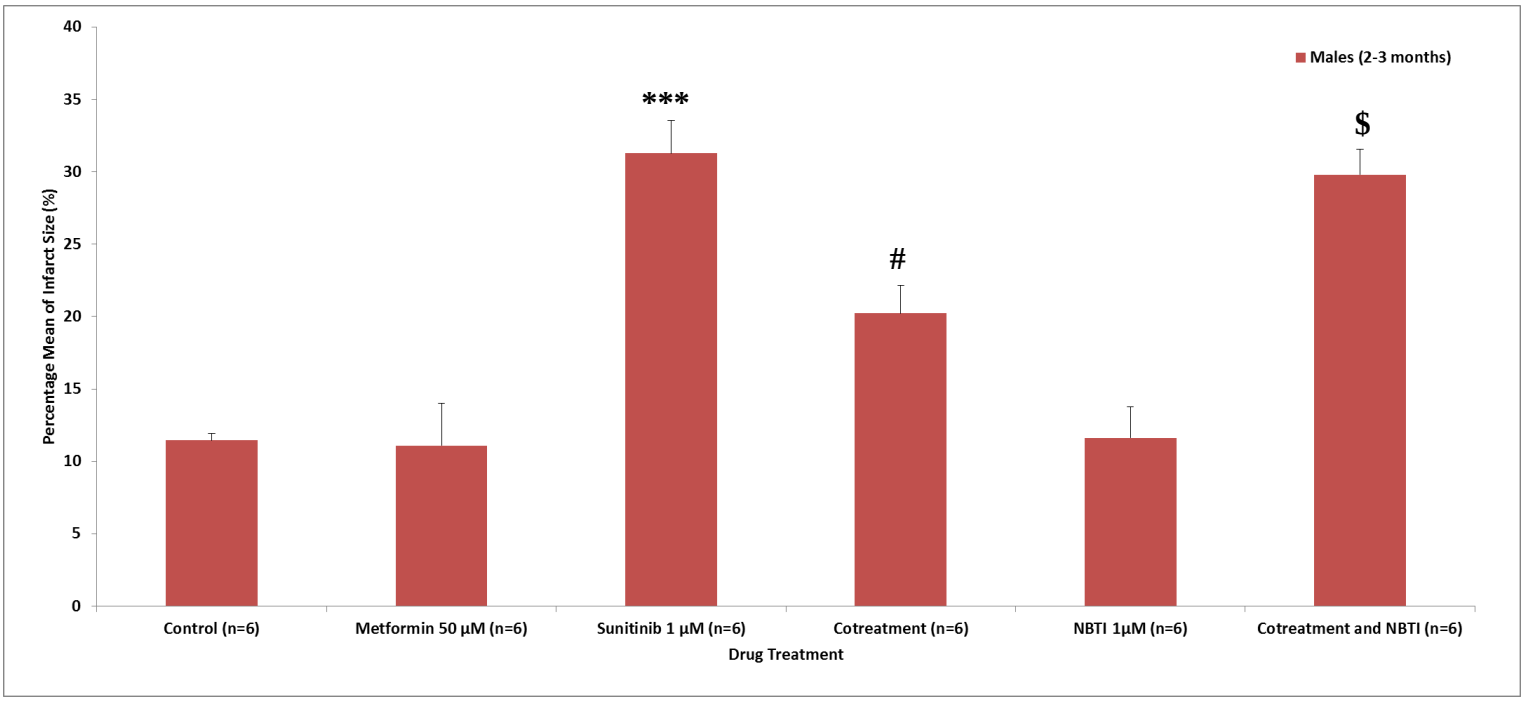

Figure 15: The effects of vehicle control, Metformin (50 µM), Sunitinib (1 µM), the combination of Metformin and Sunitinib (Co-treatment), NBTI (1 µM) and the combination of Metformin, Sunitinib and NBTI on infarct percentage (%). Data is presented as mean ± SEM of 6 experiments (n=6). *Key statistical significance: ***=p<0.001 1 µM Sunitinib vs vehicle control. #=p<0.05 Co-treatment vs Sunitinib, $=p<0.05 Co-treatment and NBTI vs Co-treatment.*

Infarct size was determined as a percentage of the total area of the heart and presented in Figure 15.

From Figure 15, it was demonstrated that the combination of Metformin, Sunitinib and NBTI (Co-treatment and NBTI) was able to attenuate the decrease in infarct percentage when compared to the co-treatment group alone (30 ± 2 % vs. 20 ± 2 %, p<0.05).

4.4. The effects of Sunitinib in the absence and presence of Metformin and NBTI on live cell population of isolated cardiac myocytes using trypan blue staining.

Results from 7 successful experiments were included for the cardiac myocyte isolation study to assess the effect on live cell population of isolated cardiac myocytes (Figure 16) during co-administration of Metformin, Sunitinib and NBTI in isolated cardiac myocytes from SD rat hearts (n=7). Experiments were carried out for vehicle control, Metformin concentration 50 µM, Sunitinib 1 µM, the combination (co-treatment) of Metformin (50 µM) and Sunitinib (1 µM), NBTI 1 µM and the combination (co-treatment and NBTI) of Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM).

Figure 16 the effects of drug treatment on live population of cardiac myocytes

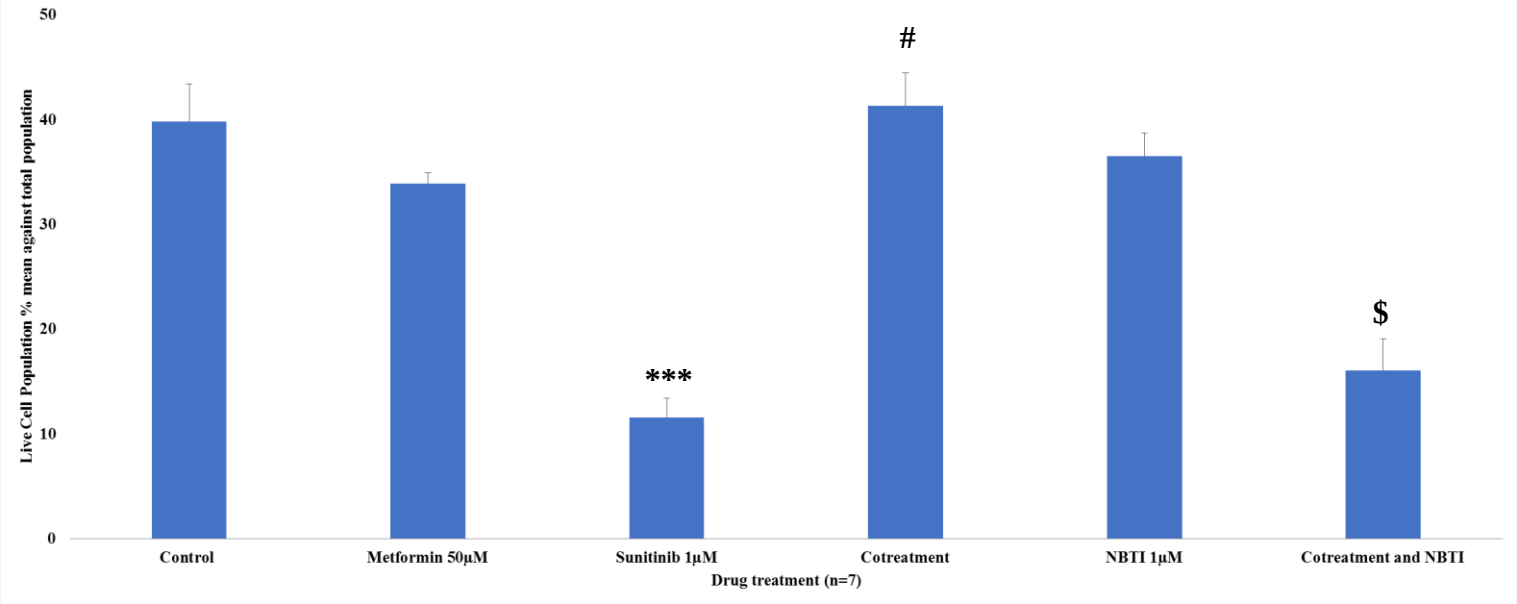

Figure 16: The effects of vehicle control, Metformin (50 µM), Sunitinib (1 µM), the combination of Metformin (50 µM) and Sunitinib (1 µM) (Co-treatment), NBTI (1 µM) and the combination of Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM) (co-treatment and NBTI) on live cell population of isolated cardiac myocytes (%) following trypan blue staining. Data is presented as mean ± SEM of 7 experiments (n=7). *Key statistical significance: ***=p<0.001 1 µM Sunitinib vs vehicle control. #=p<0.05 Co-treatment vs Sunitinib, $=p<0.05 Co-treatment and NBTI vs Co-treatment.*

Using Figure 16, live cell population was significantly decreased following Sunitinib (1 µM) administration when compared to vehicle control (12 ± 2 % vs. 40 ± 4 %, p<0.05). Moreover, the combination of Metformin (50 µM) and Sunitinib (co-treatment) was shown to attenuate the decrease in live cell population when compared to Sunitinib alone (41 ± 3 % vs. 12 ± 2 %, p<0.05). The resulting attenuation was shown to be inhibited following the introduction of NBTI in combination with Metformin and Sunitinib (co-treatment and NBTI) as demonstrated by a significant decrease in live cell population compared to the co-treatment alone (16 ± 3 % vs. 41 ± 3 %, p<0.05).

4.5. The effects of Sunitinib in the absence and presence of Metformin and NBTI in SD rat hearts using Western blot analysis for phosphorylated-AMPKα, AMPKα and GAPDH.

Results from 47 successful experiments were included for the Western blot analysis study to assess the effect for phosphorylated-AMPKα (p-AMPKα, Thr^{172}), total AMPKα and GAPDH levels during Metformin (50 μM) co-administration with Sunitinib (1 μM), and Metformin (50 μM) co-administration with Sunitinib (1 μM) and AMPK inhibitor NBTI (1 μM) during the Langendorff study. Western blot analysis was carried out to assess p-AMPKα (Thr^{172}, 62 kDa, New England BioLabs #2531), AMPKα (Total AMPKα, 62 kDa, New England BioLabs #2532) and GAPDH (D16H11, XP-(R), New England BioLabs #5174) protein levels. Samples were run alongside positive and negative controls for AMPK (AMPK Control Cell Extract, New England BioLabs #9158).

Expressed levels of p-AMPKα (Thr^{172}) were standardised to Total-AMPKα for each respective sample and presented in Figure 18. Expressed levels of p-AMPKα were standardised to house-keeping protein GAPDH for each respective sample and presented in Figure 19. Probing for p-AMPKα (Thr^{172}), Total-AMPKα and GAPDH were carried out on the same membrane and same protein samples.

Figure 17 observed Western blot image for the effect of drug treatment on p-AMPKα, Total-AMPKα and GAPDH signalling

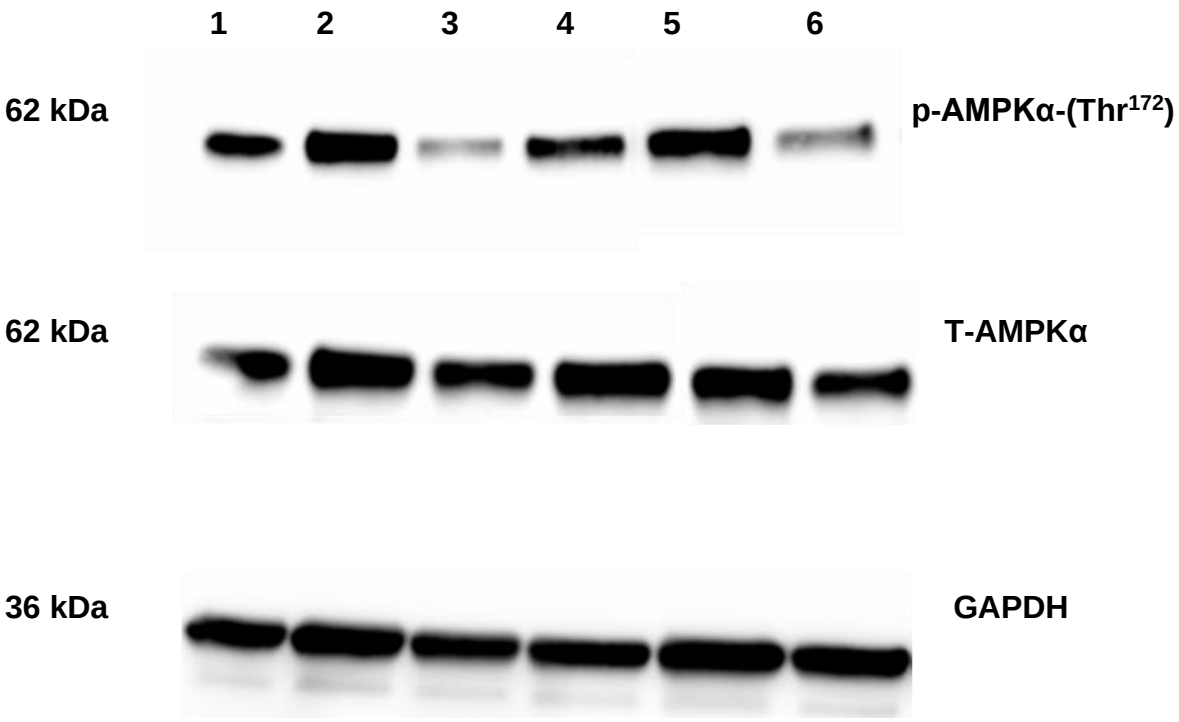

Figure 17: Western blot analysis image for phosphorylation of Thr^{172} for p-AMPKα, Total-AMPKα and GAPDH signalling for Sunitinib (1 μM) ± Metformin (50 μM) ± NBTI (1 μM) in SD rat hearts. *Key: 1=vehicle control, 2=Metformin 50 μM, 3=Sunitinib 1 μM, 4=Co-treatment (Metformin 50 μM+Sunitinib 1 μM), 5=NBTI 1 μM, 6=Co-treatment and NBTI.*

From Figure 17, bands demonstrating phosphorylation levels of p-AMPKα (Thr^{172}) for the Sunitinib treatment group (lane 3) was observed to be fainter than the vehicle control group (lane 1). In contrast, the co-treatment group (lane 4) was observed to have a darker or more intense band than the Sunitinib treatment group (lane 3). Moreover, the co-treatment and NBTI group (lane 6) was also observed to have a fainter band than the co-treatment group (lane 4).

Figure 18 Western blot analysis for the effect of drug treatment on p-AMPKα standardised to Total-AMPKα signalling

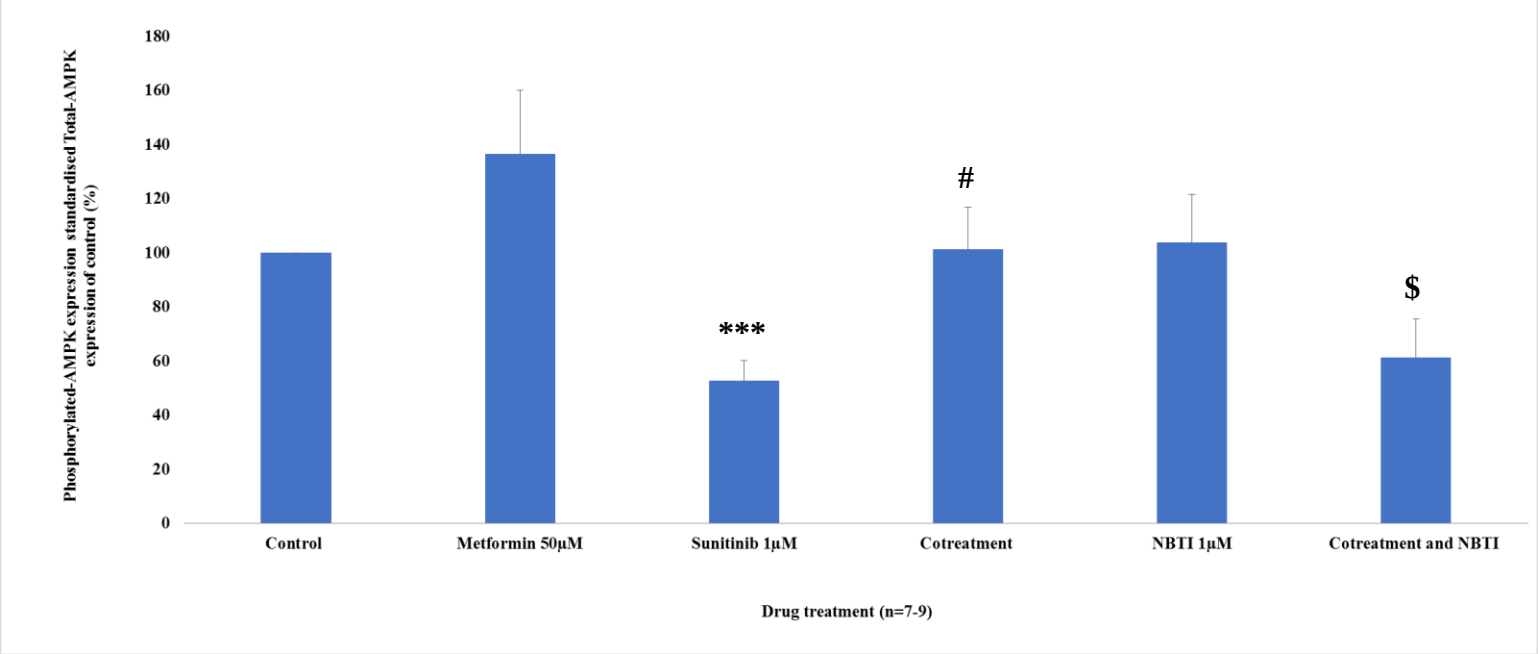

Figure 18: Western blot signalling for p-AMPKα standardised to Total-AMPKα signalling, taken for the duration of the normoxic study involving normoxic vehicle control, Metformin (50 µM), Sunitinib (1 µM), the combination (co-treatment) of Metformin (50 µM)+Sunitinib (1 µM), NBTI (1 µM) and the combination of Metformin+Sunitinib+NBTI (co-treatment and NBTI). Data is presented as mean ± SEM of 7–9 experiments (n=7–9). *Key statistical significance: ***=p<0.001 Sunitinib vs vehicle control. #=p<0.05 Co-treatment vs Sunitinib, $=p<0.05 Co-treatment and NBTI vs Co-treatment.*

From Figure 18, the Sunitinib treatment group resulted in a significant decrease in p-AMPKα signalling when compared to the vehicle control (53 ± 7 %, p<0.001), moreover the co-treatment group was demonstrated to attenuate this decrease when compared to Sunitinib treatment alone (101 ± 15 %, p<0.001). Metformin was demonstrated to increase p-AMPKα signalling when compared to the normoxic control (136 ± 24 %, p<0.001). NBTI alone demonstrated no significant changes in p-AMPKα signalling against vehicle control (104 ± 18 %, p>0.05). However, the co-treatment and NBTI group was shown to significantly decrease p-AMPKα signalling compared to the co-treatment group (61 ± 14 %, p<0.05).

Figure 19 Western blot analysis for the effect of drug treatment on p-AMPKα standardised to GAPDH signalling

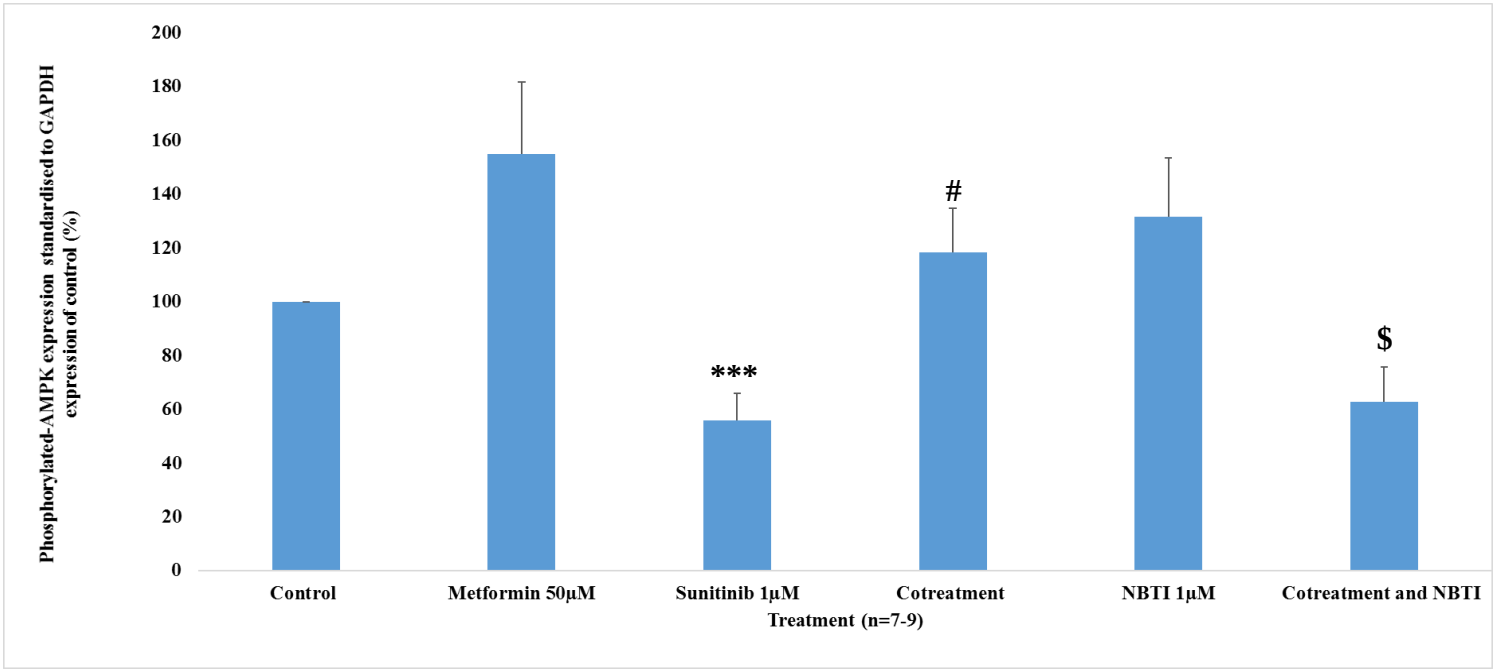

Figure 19: Western blot signalling of p-AMPKα standardised to GAPDH signalling, taken for the duration of the normoxic study involving normoxic vehicle control, Metformin 50 µM, Sunitinib 1 µM, the combination (co-treatment) of Metformin (50 µM)+Sunitinib (1 µM), NBTI 1 µM and the combination of Metformin+Sunitinib+NBTI (co-treatment and NBTI). Data is presented as mean ± SEM of 7–9 experiments (n=7–9). *Key statistical significance: ***=p<0.001 1µM Sunitinib vs vehicle control. #=p<0.05 Co-treatment vs Sunitinib, $=p<0.05 Co-treatment and NBTI vs Co-treatment.*

From Figure 19, the Sunitinib treatment group resulted in a significant decrease in p-AMPKα signalling when compared to the vehicle control (56 ± 10 %, p<0.05), moreover the co-treatment group was demonstrated to attenuate this decrease when compared to Sunitinib treatment alone (118 ± 17 %, p<0.05). Metformin was demonstrated to increase p-AMPKα signalling when compared to the normoxic control (155 ± 27 %, p<0.05). NBTI alone demonstrated a statistically significant increase in p-AMPKα signalling against vehicle control (132 ± 22 %, p<0.05, not shown). However, the co-treatment and NBTI group was shown to significantly decrease p-AMPKα signalling compared to the co-treatment group (63 ± 13 %, p<0.05).

4.6. Discussion.

Sunitinib-induced cardiotoxicity is thought to be mediated via the inhibition of several anti-apoptotic pathways as well as the activation of apoptosis itself. It was postulated in Section 3.1.-3.5., that this may have arisen form Sunitinib's ability to induce pro-apoptotic proteins and the activation of apoptosis. The exact mechanism of Sunitinib-induced cardiotoxicity is not fully understood; however to support our hypothesis the proposed activation of apoptosis may arise from the lack of selectivity of Sunitinib, reports have shown 57 of 317 kinases were shown to be competitively bound at the dose of 1 µM (Karaman *et al*. 2008, Cohen e*t al*. 2011). Furthermore, RISK has been highlighted as an important off-target kinase mediating Sunitinib cardiotoxicity (Fabian e*t al*. 2005, Anjum and Blenis 2008, Cohen *et al*. 2011), whilst AMPK was said to be implicated in Sunitinib-induced cardiotoxicity in patients (Kerkela *et al*. 2006, Chu e*t al.* 2007). Inhibition of several pathways is thought to result in the activation of cytochrome c release and therefore direct mitochondrial toxicity, whilst Sunitinib has been further hypothesised to cause toxicity via a direct interaction with the mitochondria (Fabian *et al*. 2005). To support the hypothesis, we carried out live cell counting for isolated cardiac myocytes following administration with Sunitinib. We were able to demonstrate that Sunitinib was able to significantly reduce the live cell population of cardiac myocytes when compared to the vehicle control. Studies were able to demonstrate similar results in neonatal rat ventricular cardiac myocytes however at a different concentration of 150 ng/ml (equivalent to 0.28 µM), believed to be close to the average peak plasma concentration within patients (Harvey and Leinwand 2015, Chu *et al*. 2007, Lankheet *et al*. 2011). Moreover, to support the proposed mechanism of Sunitinib-induced cardiotoxicity in Figures 13–14, the study by Harvey and Leinwand 2015 noted that Sunitinib was able to initiate a cardiotoxic response via the upregulation of apoptotic proteins Fas, Fasl, FADD, TRAIL and cleaved caspase-3, known to be involved in a pro-apoptotic response. Interestingly, the study by Henderson *et al*. (2013) was not able to determine Sunitinib-induced contractility loss and that this may not be due to cardiac myocyte damage, and the effect is not related to ischaemia caused via a reduce oxygen supply, however the concentration used was 0.1 µM, a tenth of the concentration we used. It was further indicated that investigations would be required for Sunitinib effects on ion channel, sarcoplasmic

reticulum Ca^{2+} stores and if mitochondrial function is necessary (Henderson *et al*. 2013).

Using Figure 14b, Metformin co-administration with Sunitinib was shown to significantly attenuate the reduction in LVDP caused by Sunitinib administration alone, the effects of Metformin were further inhibited when co-administered with Sunitinib and NBTI. Therefore, results from Figure 14b can suggest that the activation of the AMPK signalling pathway is occurring following Metformin administration. With this, AMPK activation was said to result in the activation of the catabolic pathway and suppress non-essential ATP-consuming processes (Kudo *et al*. 1995). The resulting activation of the catabolic pathway can contribute towards the re-distributing of energy for cardiac myocyte and myocardium function, potentially explaining the increase in LVDP following Metformin co-administration with Sunitinib (Tian *et al*. 2001, Allard *et al*. 2007, Li *et al*. 2007, Fu *et al*. 2011, Cha *et al*. 2010). As cardiac tissue requires a constant and stable source of energy, resulting in a limited reserve of ATP, by inhibiting AMPK this could disrupt the cardiac myocytes ability to meet the energy demand, possibly explaining the overall decrease in LVDP following Sunitinib administration (Kudo *et al*. 1995).

To investigate the role of AMPK signalling in Sunitinib-induced cardiotoxicity, we carried out Western blot analysis as demonstrated in Figures 17–19. We were able to demonstrate that phosphorylated levels of AMPK, at the Thr^{172} residue, was significantly decreased following Sunitinib administration at 1 μM. In accordance with our findings, phosphorylation of the α and β isoforms of ACC, a target of AMPK, is said to be reduced following Sunitinib administration, in a dose-dependent manner, after a 2 hour exposure, by Hasinoff *et al.* (2008). However, as highlighted by Mellor *et al*. (2011) the dose used (5 μM, by Hasinoff *et al.* 2008) is 25-fold greater than the therapeutic plasma concentration level. Nevertheless, ACC is a target of AMPK signalling and is required for the signalling of p-AMPKα at the Thr^{172} residue, ACC is said to be undetectable in association with the absence of Thr^{172} (Crute *et al*. 1998, Stein *et al*. 2000, Willows *et al*. 2017). Using this information, it can be suggested that our results are similar with that demonstrated by Hasinoff *et al*. (2008), of which demonstrated reduced phosphorylation of ACC following Sunitinib administration, whilst we were able to demonstrate a significant reduction

in p-AMPKα signalling following Sunitinib administration. Our results from Figures 17-19 are in agreement with results demonstrated by Laderoute *et al*. (2010), demonstrating Sunitinib-induced inhibition of p-AMPK. Moreover, Laderoute *et al*. (2010) further demonstrated Sunitinib-induced inhibition of p-ACC as well as p-AMPK following incubation of human prostate cancer cells for 4 hours. However, similar to the study by Hasinoff *et al.* (2008), the study by Laderoute used a concentration greater than that used in the Langendorff system in this study, at 5 μM and 25 μM respectively. However despite this, Western blot images and results of p-AMPK from Laderoute *et al*. (2010) are similar to results obtained in Figures 17-19. Using this information with our results, it is concluded that Sunitinib acts to potently bind to and inhibit p-AMPK signalling (Laderoute *et al*. 2010). Sunitinib is said to the AMPKα kinase domain as a type-2 protein kinase inhibitor and stabilises the catalytically inactive DFG-out conformation of the protein (Laderoute *et al*. 2010).

It has been proposed that increased phosphorylation of AMPK, before reperfusion, as well as during events of ischaemia may protect cardiac muscle from infarction, providing energy for cell viability while the myocardium is readjusting the metabolism to oxygen (Paiva *et al*. 2010, Bhamra *et al*. 2008, Gundewar *et al*. 2009, Calvert *et al*. 2008). In support of this, studies have demonstrated the extracellular activation of AMPK, such as via Metformin administration, improved myocardial energy metabolism during acute sustained ischaemic injury in non-diabetic rat hearts (Kawabata and Ishikawa 2003). Metformin-induced activation of AMPK was shown to be abolished with the non-specific nitric oxide synthase inhibitor L-NAME (Kawabata and Ishikawa 2003). Using this, in accordance with results obtained in Figures 14-18 it can be suggested that the administration of Metformin resulted in an improvement in energy metabolism, as evidenced by the restoration of LVDP similar to the vehicle control in Figure 14b. Moreover, the inhibition of AMPK signalling resulted in an attenuation of the LVDP restoration in Figure 14b. To support this, acute ischaemia-reperfusion studies suggest AMPK may modulate PPAR-α directly or indirectly through PGC-1α, as PPAR-α was indicated to be attributed to a post-translational level (Barreto-Torres *et al*. 2012). In normal conditions the myocardium uses fatty acid synthesis of glucose oxidation as the main source of energy, fatty acid oxidation is transcriptionally regulated via

members of nuclear receptor superfamily such as PPAR and their coactivator PGC-1α (Vega *et al*. 2010, Mu *et al*. 2001, Schreiber *et al*. 2003). Furthermore, it is said that AMPK inhibits biosynthetic pathways via inhibition of mTOR and Co-A carboxylase, whilst many studies have used pharmacological AMPK activators Metformin or AICAR to initiate AMPK activation.

It is known that the primary mechanism responsible for AMPK activation involves phosphorylation at the Thr^{172} residue found within the activation loop of the α-catalytic subunits (Stein *et* al. 2000, Baron *et al*. 2005, Carling *et al*. 2003). The phosphorylation of Thr^{172} is mediated by the upstream kinase, AMPK-activating protein kinase (AMPKK) (Hawley *et al*. 1995). Moreover, during ischaemic stress AMPKK was shown to be activated in the heart via AMP augmentation to phosphorylate and activate the AMPK α subunit and interacting with the heterotrimeric AMPK complex (Baron *et al*. 2005). Furthermore, the use of AICAR was able to demonstrate the interaction of nucleotides with heterotrimeric AMPK was sufficient to increase AMPK Thr^{172} phosphorylation in *in-vivo*, in the absence of AMPKK (Baron *et al*. 2005). For this, we aimed to demonstrate the potential for AMPK phosphorylation to be affected by Metformin and Sunitinib treatment by measuring the levels of phosphorylation at the Thr^{172} as shown in Figures 18-19. We demonstrated that Metformin administration at 50 μM was able to significantly increase phosphorylation of AMPK at the Thr^{172} residue against vehicle control, in Figures 18 and 19, when standardised to both total AMPK and GAPDH. The observed results were expected particularly as Metformin acts to increase AMPK signalling (Rena *et al*. 2013). In regards to Thr^{172} phosphorylation, our results are in accordance to existing studies demonstrating a decrease in p-AMPK signalling following Sunitinib treatment, whilst p-AMPK signalling is increased with Metformin administration (Laderoute *et* al. 2010, Cohen *et al*. 2011, Zarrouk *et al*. 2014, Chu *et al*. 2007, Kerkela *et al*. 2009).

We previously suggested and proposed, in Section 3.1.-3.5., the potential for Sunitinib to inactivate or reduce phosphorylation of AMPK signalling as well as other signalling pathways. It was demonstrated in Figures 18 and 19 that Sunitinib administration at 1 μM was able to significantly decrease phosphorylation of AMPK against the vehicle control. Moreover, the co-administration of Metformin with

Sunitinib was able to attenuate this decrease in AMPK as demonstrated. The introduction of NBTI with the co-administration of Metformin and Sunitinib was able to attenuate the effects of the co-treatment. This was demonstrated following the significant decrease in AMPK phosphorylation at the Thr^{172} residue compared to the co-treatment group in Figures 18 and 19 following Western blot analysis. The observed results suggest that the use of NBTI was able to inhibit or prevent the activation of AMPK by Metformin, following Sunitinib-induced cardiotoxicity. The results demonstrated can be said to be similar to that proposed by Paiva *et al*. (2010) and the results demonstrated by Aymerich *et al*. (2006).

The use of NBTI at 1 μM was able to inhibit ENT-related transport, in support of this the study by Aymerich *et al*. (2006) demonstrated an approximate 80% decrease in ENT-transport activity and adenosine transport following incubation with NBTI in cultured cells. The authors concluded that extracellular adenosine was able to activate AMPK via a mechanism requiring adenosine transport, resulting in an increase in the AMP intracellular pool (Aymerich *et al*. 2006). Moreover, the same effect was demonstrated when blocking adenosine transport via CNT2, resulting in the inhibition of the AMPK signalling pathway, whilst the addition of adenosine demonstrated a significant increase in AMPK signalling (Aymerich *et al*. 2006). AMP generation was shown to be required for AMPK activation, with a marked decrease in ADP concentration, following the addition of adenosine (Aymerich *et al*. 2006). The study further demonstrated the inhibition of increased intracellular AMP concentrations after adenosine addition, and a marked decrease in ADP concentration, the authors concluded that the generation of AMP from extracellular adenosine was required for AMPK activation (Aymerich *et al*. 2006). To support this, Aymerich *et al*. (2006) demonstrated the addition of extracellular adenosine was said to enhance ACC phosphorylation, which was otherwise abolished following CNT2 inhibition. The mechanism of AMPK inhibition via NBTI was summarised using Figure 10. Using this information, extracellular adenosine, via administration with Metformin, was required for the activation and phosphorylation of AMPK Figures 18-19, therefore inhibition of AMPK signalling via this route resulted in the inhibition of Metformin-induced AMPK activation, hence the results obtained following administration of NBTI with the combination of Metformin and Sunitinib. As we discussed that AMPK signalling may be a primary route of Metformin-induced

cardioprotection, the inhibition of AMPK resulted in the loss of the cardioprotective properties in Figures 14b, 15, 16, 18 and 19. This was evidenced by the increase in infarct percentage as demonstrated in Figure 15 for the co-treatment and NBTI group compared to the co-treatment group alone.

However, it must be noted that several non-specific or off-target mechanisms of cardioprotection could be taking place during Metformin administration. As explained, Metformin is proposed to inhibit mitochondrial complex I and AMP deaminase thereby increasing the AMP:ATP ratio and activating AMPK, this results in the phosphorylation of eNOS involved in the RISK pathway as explained by Bromage and Yellon 2015. The RISK pathway is known to inhibit the opening of mPTP which initiates the effects of calcium influx and ROS generation during reperfusion following the initiation of ischaemia (Bromage and Yellon 2015). Metformin has been noted to also exert pleiotropic effects such as changes in blood rheology, serum lipid profile as well as anti-ischaemic effects (Anfossi *et al*. 2010). However, the exact mode of cardioprotection by Metformin is still not fully understood. Metformin is suggested to increase activity of NO-synthase, resulting in a vasoprotective response (Sartoretto *et al*. 2005, Bhalla *et al*. 1996), furthermore Metformin was shown to attenuate LV post-ischaemic dysfunction (Legtenberg *et al*. 2002). Using this in regards to our results obtained in Figure 14b, it was demonstrated that Metformin was able to attenuate the Sunitinib-induced decrease in LVDP, an effect that was attenuated following the introduction of NBTI, supporting our evidence that Metformin administration resulted in an improvement in LV activity, associated with the activation of the AMPK signalling pathway.

In accordance with our evidence of AMPK activation following Metformin co-administration, Metformin is indicated to achieve a plasma concentration of 10-20 μM/l in patient studies and was able to demonstrate a significant activation of AMPK in H4IIE cells at a Metformin concentration 50 μM/l, whilst activation was said to be two-fold greater following 72 hours of incubation (Hawley *et al*. 2002). Moreover, the authors concluded that higher doses of Metformin are not required for maximal AMPK activation to achieve a beneficial therapeutic effect (Hawley *et al*. 2002). Furthermore, patient studies involving greater doses of Metformin were said to result in gastrointestinal side effects, whilst also posing a risk for lactic acidosis,

possibly resulting from the compound acting as an inhibitor of the respiratory chain at higher concentrations (Hawley *et al*. 2002, Owen e*t al*. 2000). Taking these factors into consideration the relevant dose of 50 µM was agreed to be used in the adjunctive treatment. Following this, we demonstrated that Metformin at 50 µM was able to significantly increase p-AMPKα signalling compared to the vehicle control, and a similar result was observed when administered together with Sunitinib when compared to Sunitinib treatment alone.

Our results showed that Metformin induced ~~s~~ a cardioprotective response. To support this, Spector e*t al*. (2007) demonstrated that AMPK activation leads to the protection against TNFα-induced cell death and activation of AMPK is able to reduce associated cardiotoxicity. HER2 small molecule tyrosine inhibitors demonstrated that inhibition of HER2 and EGFR kinase activity via GW2974 prevention of HER3 transactivation, leading to increased intracellular Ca^{2+} (Spector *et al*. 2007). The increase in intracellular Ca^{2+} is said to lead to activation of CAMKK, CAMKK in turn activates AMPK and the activation of AMPK inhibits mTOR, eEF2, ACC blocking protein and fatty acid synthesis, whilst AMPK activation induces fatty acid oxidation via activation of mitochondrial ERRα and PGC-1α (Spector *et al*. 2007).

In accordance with our results in Figure 16 demonstrating that Metformin was able to attenuate Sunitinib-induced cardiac myocyte cell loss, Wang *et al*. (2017) demonstrated that Metformin was able to prevent ischaemia-reperfusion-induced apoptosis of cardiac myocytes; consequent AMPK activation was demonstrated with the Metformin treated group when compared to the normoxic control. Moreover within *in-vitro* AICAR was used to demonstrate the same AMPK activation whilst this was abolished using Compound C, highlighting the role of AMPK in Metformin-induced cardioprotection (Wang *et al*. 2017). AMPK activation is known to occur following phosphorylation at the Thr^{172} and the α subunit via upstream AMPK kinases such as the calcium-activated protein kinases CAMKKβ and LKB1 (Mercy *et al*. 2005, Lizcano *et al*. 2004, Shaw *et al*. 2004, Inoki *et al*. 2003, Spector *et al*. 2003). Within the heart, as well as skeletal muscle, AMPK activation results in the phosphorylation and inhibition of ACC, this acts to reduce malonyl-CoA levels which reduces the inhibition of CPT-1, the depression of CPT-1 results in the increase in β-oxidation of fatty acid and results in the increased mitochondrial production of

ATP (Spector *et al*. 2007, Kudo *et al*. 1995, Kudo *et al*. 1996, Lehman *et al*. 2000). Furthermore, upon stress-activation of AMPK, the resulting cascade is known to inhibit protein synthesis of mTOR and directly modulating elongation factor (EFF) 2, this translation elongation factor is considered to be associated with cardioprotective properties (Spector *et al*. 2007, Bolster *et al*. 2002, Horman *et al*. 2002, Terai *et al*. 2005). Moreover AMPK-mediated TSC2 phosphorylation inhibition of cap-dependent translation is considered to be critical in cell survival in response to ATP depletion (Spector *et al*. 2003, Inoki *et al*. 2005).

The use of the Langendorff system has been used to monitor LV contractility whilst also measuring ECG changes and the use of the isolated heart allows for the measuring of multiple indices of cardiac function, such as measuring biopsied tissue for protein or genomic biomarkers for cardiac injury and stress in a single assay allows for the reduction in animal use and experimental costs (Henderson *et al*. 2013). In our study, we found that hearts perfused with KH buffer and vehicle remained stable with very slight changes over the 155 minute protocol. Reductions in HR, and possibly CF, within the control groups can be attributed to run down during KH buffer perfusion, an effect described to occur due to inefficient energy production (Henderson *et al*. 2013). It is said that for energy production pyruvate and glucose, provided via KH buffer, is less favoured than fatty acid use or insulin perfusion by the heart which may contribute towards a better stability and longevity within the Langendorff system (Henderson *et al*. 2013, Goodwin *et* al. 1998, Lopaschuk and Saddik 1992, Watanabe *et al*. 1984).

To date, current studies involving AMPK activation in Sunitinib-induced cardiotoxicity studies have focused on the pre-treatment of cardiac tissue with either Metformin or AICAR and have thus far failed to demonstrate potential cardioprotection via pre-treatment (Hasinoff e*t al*. 2008, Kerkela e*t al*. 2009, Cohen e*t al*. 2011). However, it must be noted that the study by Hasinoff *et al*. (2008) involved a pre-treatment period with Metformin, less that of the incubation period with Sunitinib. Moreover, the authors failed to demonstrate if Sunitinib-inactivation of AMPK can be overcome during adjunctive treatment with Metformin. Likewise the study by Cohen e*t al*. (2011) involved pre-treating stem cell-derived cardiac myocytes with either Metformin or AICAR for a period of 3 hours, compared to the

24 hour incubation with Sunitinib to measure LDH levels. This could indicate the potential for Sunitinib to inactivate AMPK signalling following the removal of Metformin or AICAR, highlighting the importance of investigating AMPK-activation via adjunctive treatment rather than a means of prior activation of AMPK, as evidenced by the study by Laderoute *et al*. (2010) demonstrating Sunitinib potently binding to AMPK. Moreover, it was noted that LDH release as an indicator of cell death may not be suitable to indicate ATP content in cardiac myocytes for Sunitinib-induced cardiotoxicity (Cohen *et al*. 2011). To date, no study has investigated the re-activation of AMPK after Sunitinib treatment, in order to investigate the potential for Metformin to activate AMPK signalling to counter Sunitinib's ability to inhibit AMPK signalling. In contrast to many existing studies, our study aimed at investigating potential AMPK activation via adjunctive administration with the indirect AMPK-activator Metformin rather than pre-treatment. An overexpression of a constitutively active mutant form of AMPK was shown to be able to reduce Sunitinib-mediated apoptosis, whilst AMPK activation in the heart was shown to demonstrate anti-apoptotic properties, highlighting the potential need for AMPK activation to be present during Sunitinib administration and not via pre-treatment (Kerkela *et al*. 2009, Hickson-Bick e*t al*. 2000, Russell *et al*. 2004, Shibata *et al*. 2005, Terai e*t al.* 2005, Dyck and Lopaschuk 2006, Spector *et al*. 2007).

In our study we used the inhibition of AMPK via the use of NBTI. To date the most widely accepted cell permeable AMPK-inhibitor for inhibiting Metformin-induced AMPK activation is Dorsomorphin Dihydrochloride (Compound C, 6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrzolo [1, 5-a] pyrimidine) (Zhou *et al*. 2001, Isakovic *et al*. 2007, Tang *et al*. 2011). Dorsomorphin was demonstrated to be a potent and reversible AMPK inhibitor that is competitive with ATP with K_i=109 ± 16 nM in the absence of AMP (Zhou *et al*. 2001). Moreover, Dorsomorphin, structurally related to TK receptor inhibitors (Fraley *et al*. 2002), did not significantly inhibit structurally related kinases ZAPK, SYK, PKC, PKA and JAK3 (Zhou *et al*. 2001) however was demonstrated to inhibit bone morphogenic protein (BMP) signalling via an AMPK-independent manner (Paul *et al*. 2008). Furthermore, Dorsomorphin was also shown to reduce cell proliferation and growth of astrocytic tumours in *in-vitro* and *in-vivo* (Rios *et al*. 2013) and was able to inhibit AMPK activity and kill glioma cells (Liu *et al*. 2014). It was suggested that Dorsomorphin not be used to

investigate AMPK functions due to the compound alone being significantly cytotoxic via an AMPK-independent route, whilst also reported to inhibit kinases with a lower Km that that of AMPK, the study by Bain *et al*. (2007) identified that a concentration of above 40 μM (EC_{50}=0.1–0.2 μM) is required to inhibit AMPK completely in culture media, thereby the authors have advised against using the compound to inhibit AMPK (Bain *et al*. 2007, Vogt *et al*. 2011, Jin *et al*. 2009). Following this information, we favoured the use of the ENT-inhibitor NBTI in order to potentially prevent the activation of AMPK, over the use of Dorsomorphin. Moreover, in a study with glioma cells, Dorsomorphin was demonstrated to induce caspase-3 mediated apoptosis (Liu *et al*. 2014), whilst in breast cancer cells Dorsomorphin resulted in ceramide production and redistribution of Bax from the cytoplasm to the mitochondria, both effects were said to be AMPK-independent (Jin *et al*. 209). Finally, it has been suggested that Dorsomorphin works by blocking total AMPK activity rather than phosphorylation of AMPK, indicating this to be difficult to assess when administered in the context of Metformin use in AMPK activation (Paiva *et al*. 2010, Leon *et al*. 2014). As demonstrated by band intensity in Figure 16, total levels of AMPKα are not altered during treatment of Metformin, Sunitinib or both in co-administration, but rather phosphorylation of AMPK is altered between treatment groups, shown in Figure 18 and 19, indicating that the use of Dorsomorphin would not be relevant in this study.

It has been suggested that Metformin's cardioprotective properties could be independent of hypoglycaemic actions (Solskov *et al*. 2008). Within our study we have primarily focussed on the AMPK signalling pathway, it is worth noting that drugs activate different pathways when administered acutely compared to chronically (Mensah *et al*. 2005, Mocanu and Yellon 2007, Teresi *et al*. 2006). Knowing this, Metformin is said to have an effect on numerous endogenous factors associated with cell survival including peroxisome PGC-1α known to have a role in energy metabolism and is expressed on mitochondrial-rich tissue such as the heart (Suwa *et al*. 2006, Liang *et al*. 2006). An increase in AMPK and PGC-1α were shown to exert a beneficial effect on cardiac function within a HF model (Gundewar *et al*. 2009). The study by Saeedi *et al*. (2008) demonstrated that Metformin induced metabolic changes in the heart in the absence of AMPK in H9c2 cells derived from rat embryonic cardiac myocytes. The authors demonstrated that the metabolic

effects of Metformin were abrogated during the pharmacological inhibition of p38 MAPK and PKC. ACC is down-stream of AMPK and phosphorylation of ACC was shown to not differ between Metformin-treated and non-treated hearts and H9c2 cells. With the knowledge that a reduction in cellular energy status following Metformin administration likely occurs via Metformin's ability to inhibit complex I of the respiratory chain complex (Hardie 2006, El-Mir e*t al*. 2000, Owen *et al*. 2000), Saeedi *et al*. (2008) failed to demonstrate an impairment in cellular energy status and suggested that this was an indicator of AMPK not being activated, hence the lack of reduction in cellular energy status. It was said that inhibition of PKC and p38 MAPK resulted in the reduction in Metformin acceleration of glycolysis in H9c2 cells, highlighting the importance of the two pathways. However, it is worth noting that H9c2 cells are rat embryonic ventricle derived and although morphological characteristically similar to immature embryonic cardiac myocytes and biochemically and electrophysiologically similar to adult cardiac cells, such cells are not completely representative of cardiac myocytes. For this, we favoured the use of isolated cardiac myocytes.

Similar to our results, the study by Cohen *et al*. (2011) demonstrated that Sunitinib treatment resulted in cardiac myocyte death when treated for 2 hours. However, the concentration of Sunitinib used by Cohen *et al*. (2011) was 15 times greater than the concentration we used for the Langendorff, Western blot analysis and isolated cardiac myocyte studies. Cohen *et* al. (2011) also investigated levels of LDH release, whereas we measured live cell populations of isolated cardiac myocytes. Moreover, similar to our results, phosphorylation of AMPK at the Thr^{172} residue was almost absent for Sunitinib, when compared to vehicle control (Cohen *et al*. 2011). In contrast this study, we demonstrated similar results using Sunitinib at 1 µM but during a 4 hour treatment period. For this, we aimed to demonstrate the effect of Sunitinib directly on isolated cardiac myocytes using the same concentration of Sunitinib that we used in the Langendorff experiment. A concentration of 15 µM for Sunitinib would not be suitable in the Langendorff study as this could result in a complete death of the isolated rat heart.

Moreover, treatment with Metformin alone was able to significantly increase levels of p-AMPK, compared to vehicle control (Cohen *et al*. 2011). However, Cohen *et al*. (2011) failed to demonstrate any cardioprotection of Metformin. It must be said that

the study involved pre-treating cardiac myocytes with Metformin, in comparison to our study that involved the co-administration of Metformin with Sunitinib. Moreover Cohen *et al*. (2011) measured LDH release in comparison to the live cell count for our study. LDH release may not be a predictive measure for ATP content, upon activation via metabolic stress AMPK acts to maintain ATP levels (Dyck and Lopaschuk 2006, Cohen *et al*. 2011), therefore the results obtained by Cohen e*t al*. (2011) may not be the same when used in an *in-vivo* or *ex-vivo* experimentation. Moreover Cohen *et al*. (2011) indicated that ATP depletion was recorded as 80%, as well as a significant increase in apoptosis with 31 μM Sunitinib, whilst pre-treatment with Metformin during 15 μM Sunitinib treatment was not able to attenuate cardiotoxicity or decrease phosphorylating levels of AMPK in Western blots. In contrast, we were able to demonstrate that the clinically relevant dose of 1 μM Sunitinib was able increase infarct percentage in SD rat hearts, whilst the non-toxic dose of Metformin 50 μM was able to attenuate Sunitinib-induced toxicity. It is difficult to foresee how the dose concentrations used by Cohen *et al*. (2011) were to be replicated in our *ex-vivo* experimentation due to the high concentrations of Sunitinib potentially causing a greater percentage of infarction and over-riding any potential cardioprotection offered by Metformin, however this would need to be investigated further. This is particularly important as the authors concluded that AMPK reactivation was not able to attenuate caspase-3/7 cleavage and did not result in a “rescue” of ATP, therefore it was said that AMPK alone was not a critical protein that contributed towards Sunitinib-induced cardiotoxicity (Cohen e*t al*. 2011).

It is also worth noting that Cohen *et al*. (2011) demonstrated that treatment of human iPSC-CM whole cell lysates with 15 μM Sunitinib and 31 μM Metformin inhibitor RO-3857 did reduce the phosphorylation status of pACC-Ser79, a down-stream target of AMPK. Moreover, in a previous set of data Cohen *et al*. (2011) demonstrated that 15 μM Sunitinib reduced the phosphorylation status of p-AMPKα (Thr^{172}), the opposite was demonstrated with Metformin and AICAR treatment, and however as mentioned previously the cells used were pre-treated with either Metformin or AICAR but not used in coadministration with Sunitinib. In contrast, our study attempts to demonstrate that coadministration of Sunitinib and Metformin is able to prevent the reduction in the phosphorylated status of AMPK due to Sunitinib-induced toxicity.

In contrast to the results obtained by Cohen e*t al*. (2011) and Hasinoff *et al*. (2007), Metformin was able to reduce infarction percentage when administered at the first 15 minutes of reperfusion (Paiva *et al*. 2010). The study by Paiva *et al*. (2010) demonstrated that the protective effect of Metformin, via AMPK activation, was not abolished following Compound C when administered 5 minutes after the administration of Metformin at the onset of reperfusion, this suggests that the phosphorylation of AMPK was not affected once Metformin-induced phosphorylation of AMPK was activated. However, as the study focussed on ischaemia-reperfusion it must be noted that other signalling pathways may be involved. If Sunitinib is able to reduce AMPK signalling, our data shows that Sunitinib was not able to inhibit or decrease phosphorylation of AMPK once Metformin was introduced and continuously perfused. Moreover, our data suggests that Metformin was able to activate AMPK during the state of Sunitinib-induced cardiotoxicity as evidenced by p-AMPK levels in Figures 18 and 19. This was similar to results obtained by Paiva *et al*. (2010) when investigating AMPK activation at the onset of reperfusion. Data regarding Metformin and *ex-vivo* animal experiments to demonstrate effects on HR, LVDP and CF are limited and sometimes difficult to conclude. It is worth noting that within settings of ischaemia-reperfusion, the study by Paiva *et al*. (2010) demonstrated that Metformin did not improve LVDP and did not have any significance when administered prior to and at the onset of reperfusion, despite demonstrating a reduction in infarct percentage (Paiva *et al*. 2010).

Despite our data demonstrating Metformin has cardioprotective properties, considerations need to be taken when administering Metformin use in animal-based models. As we have previously stated, our selected dose of Metformin is higher than the suggested steady state plasma levels administered, the steady state plasma levels of Metformin are said to be approximately 10-40 µM (Cusi and DelFronzo 1998, Wiernsperger 1999, Sum *et al*. 1992), whilst studies involving rats have demonstrated that levels in liver are considerably higher than plasma (Wilcock *et al*. 1991). Moreover, membrane permeability is considered to be time-dependent whilst Metformin-activated AMPK signalling was indicated to be concentration and time-dependent (Owen *et al*. 2000, Zhou *et al*. 2001). It was suggested that high concentrations or long-term exposure to Metformin is required to see the effects of AMPK (Zhou *et al*. 2001). The study by Yang and Holman (2006) indicated the

activation of AMPK via Metformin increases glucose uptake in cardiac myocytes at a concentration of 1 mM during an 18 hour course of treatment. However, in contrast the study by Bertrand *et al*. (2006) failed to demonstrate the same effects with the same concentration during a 4-hour treatment but still increased glucose uptake, however exposure to concentrations 5 and 10 mM for 4 hours did result in an activation of AMPK and the stimulation of glucose uptake.

When investigating ischaemia, it has been highlighted that ischaemia-induced AMPK activation increases glucose uptake as well as anaerobic utilisation, this results in the heart producing sufficient ATP in order to maintain cardiac function (Paiva *et al*. 2010, Dyck and Lopaschuk 2006). This would suggest that during ischaemia, particularly Sunitinib-induced ischaemia via necrosis, the myocardium acts to activate AMPK to maintain cardiac function, this was particularly highlighted in Figures 17, 18 and 19, as we demonstrated some phosphorylation of AMPK occurred during Sunitinib administration rather than total inhibition of AMPK as suggested by Force e*t al*. (2007). Moreover, our results are in accordance with results shown by Laderoute *et* al. (2010), of which Sunitinib resulted in an inhibition of p-AMPKα but did not result in a complete inhibition of AMPK signalling as evidenced on Western blot analysis. However, the activation of AMPK via the myocardium is suggested to not be enough to counter Sunitinib's proposed other routes of cytotoxicity involving RSK and Akt, as well as pro-apoptotic proteins (Chu *et al*. 2007, Cohen *et al.* 2011).

It is known that Metformin does not directly act on LKB1 or AMPK but rather indirectly via acting upon the respiratory chain complex 1 (El-Mir *et al.* 2000, Owen *et al*. 2000), something that is considerably misunderstood amongst many articles. It is understood that inhibition of ATP synthesis results in a rise in the ADP: ATP ratio, which is said to be amplified via adenylate kinase and results in a larger rise on AMP: ATP (El-Mir *et al.* 2000, Owen *et al*. 2000, Hardie 2006). The binding of AMP to AMPK allows for the better substrate binding for LKB1, whilst acting as a worse substrate for competing protein phosphatase, the resulting complex is said to be sensitive enough for a small increase in AMP: ATP to produce a large change in AMPK activity (Hardie *et al*. 1999, Scott e*t al*. 2004). Metformin was demonstrated to not significantly phosphorylate AMPK via LKB1 in cell-free assays (Hawley *et al*. 2002), however as LKB1 is required for Metformin to activate AMPK in cultured cells

and *in vivo* in skeletal muscle the activity of LKB1 is considered to not be altered during treatment of Metformin (Lizcano *et al*. 2004, Sakamoto *et al*. 2004, Hawley e*t al*. 2003, Sakamoto *et al*. 2005). Furthermore, AMPK-related kinases downstream of LKB1 were demonstrated to not be activated during Phenformin cultured isolated rat muscle during conditions of AMPK activation via phosphorylation at the LKB1 site, although LKB1 is required for biguanides such as Metformin to activate AMPK it is said that LKB1 is not the direct target (Sakamoto e*t al*. 2004).

4.7. Conclusion.

To conclude, data presented in this chapter demonstrated that the associated cardiotoxicity following Sunitinib administration using the Langendorff model and isolated cardiac myocytes including changes in haemodynamics, infarct percentage and live cell population, were attenuated when co-administered with Metformin. Moreover, the resulting cardioprotection was shown to be inhibited by the use of NBTI, resulting in the suggesting inhibition of adenosine binding to hENT1, thereby resulting in the attenuation of AMPK signalling. To confirm this, western blotting was carried and was able to successfully demonstrate that p-AMPK signalling was significantly decreased following Sunitinib administration. Furthermore, levels of p-AMPK signalling were shown to be restored followed co-administration with Metformin, this was confirmed using NBTI, suggesting the involvement of AMPK signalling in Metformin-induced cardioprotection.

Chapter Five: The effect of Metformin co-administration during Sunitinib-induced cytotoxicity in cancer cell lines.

Data and contents in this chapter were published / presented as following:

Conferences

- British Pharmacological Society Annual Conference (2018) London, England.
- 2nd MCRN meeting at Aston University, (2018), Birmingham, England.
- British Society of Cardiovascular Research Autumn Meeting (2017), Oxford, England.
- Safety Pharmacology Society and National Centre for the Replacement, Refinement and Reduction of Animals in Research: The use of human tissues for safety assessment, Regional meeting (2017), Coventry, England.

Abstract and poster presentation

- The Role of AMPK Signalling During Metformin Co-administration with Sunitinib, British Pharmacological Society Annual Conference (2018), London, England.
- The Role of AMPK Signalling During Metformin Co-administration with Sunitinib, 2nd MCRN meeting at Aston University, (2018), Birmingham, England.
- *The Assessment of the Cardioprotective Properties of Metformin during Sunitinib-Induced Cytotoxicity,* British Society of Cardiovascular Research Autumn Conference (2017), Oxford, England.
- *The Cardioprotective Properties of Metformin during Sunitinib-Induced Cytotoxicity*, Safety Pharmacology Society and National Centre for the Replacement, Refinement and Reduction of Animals in Research: The use of human tissues for safety assessment, Regional meeting (2017) Coventry, England.

5.1. Introduction.

As discussed in Chapter 1, the multi- TKI Sunitinib is currently prescribed as a first-line agent for the treatment against certain cancers such as renal-cell carcinoma in selected patients (Motzer *et al*. 2007, Powles *et al*. 2011). However, reports have highlighted co-morbidities associated with Sunitinib treatment. Chemotherapy itself and the release of cytokines have been associated with contributing towards developing co-morbidity in patients undergoing cancer treatment, including systolic dysfunction, reductions in ejection fraction and myocardial stunning contributing towards ischaemic heart disease (van Haelst *et al*. 2006, Yang *et al*. 2010, Goede *et al*. 2014). The concept that cancer is not only a potentially curable disease but also a manageable disease has been described, with small molecule TKIs playing a vital role treatment (Force *et al*. 2007). Co-morbid diseases are described to be aggressive and pose a greater risk to patients than the malignancy, therefore prevention and treatment are vital (Oeffinger e*t al*. 2006, Force e*t al*. 2007). However, complications exist regarding cancer treatment, cardiovascular disease in particular is said to be a greater risk in young cancer survivors of whom have been treated with chemotherapeutic agents (Oeffinger *et al*. 2006). Therefore, attention has focussed on TKIs, particularly humanised monoclonal antibodies and small-molecule inhibitors against tyrosine kinase receptors (Krause and Van Etten 2005), as summarised in Table 1, Chapter 1 Section 1.9.

It has been mentioned that clinical trials involving certain chemotherapeutic agents do not include predefined cardiac endpoints, therefore the measuring of LV function before and during treatment as well as the rate of cardiotoxicity associated with TKIs are not known (Force *et al*. 2007). The review by Force *et* al. (2007) mentioned that cardiotoxicity is not a TK class effect, and that toxicity is uncommon with TKIs such as those targeting EGFR, therefore toxicity needs to be determined on a case–by-case basis (Force *et al*. 2007). Moreover, the same publication mentioned that it is difficult to diagnose coronary HF in cancer patients, particularly as many possible explanations exist regarding the development of dyspnoea, fatigue, and oedema other than LV dysfunction, all symptoms of coronary HF (Force *et al.* 2007). Furthermore, Force *et al*. (2007) highlighted that even when assessments of LV dysfunction have been included in clinical trials, rates of LV dysfunction are being

reported to make it difficult to examine the finding in a meaning context, rates of HF determined before drug approval are excluded and clinical trials are typically short of duration, whereas therapy with certain agents may be lifelong.

Using this information, the management, treatment and prevention of co-morbidities such as cardiovascular side-effects are crucial for patients undergoing cancer treatment. We have previously demonstrated in Chapter 3 and 4 that Sunitinib, when administered at the relevant concentration of 1 μM in the Langendorff system with rat hearts, cardiotoxicity was observed such as increasing infarct percentage, changes in haemodynamics and the loss of a live cell population of isolated cardiac myocytes. We further demonstrated that the co-administration with the anti-diabetic agent Metformin was able to attenuate the associated cardiotoxic effects of Sunitinib. Following this, in Chapter 4 we investigated the route of the associated effects by investigating the role of the AMPK signalling pathway. We demonstrated that Sunitinib was able to decrease phosphorylated levels of AMPK signalling in Western blot analysis, an associated effect that is hypothesised as an anti-neoplastic activity for Sunitinib treatment (Force *et al*. 2007, Laderoute *et al*. 2010), however the co-administration of Metformin was able to attenuate the decrease in p-AMPKα signalling. With this, it needs to be examined whether the effect of Metformin co-administration is preventing Sunitinib's ability to carry out its mode of action in cancer treatment.

The role of AMPK is implemented in Metformin's ability to inhibit mTORC1, directly inactivating the Ragulator complex, thereby inhibiting RAG, GTPases and disassociating mTORC1 from the activator RHEB (Long *et* al. 2005, Mahoney *et* al. 2018, Pernicova and Korbonits 2014). mTORC1 signalling was shown to be activated through RAG GTPases, recruiting mTORC1 to the lysosomal surface for the activating by RHEB (Kalender *et al*. 2010, Efeyan *et al*. 2013). Activation of the Ragulator mechanism is described to be responsive to perturbations in energy status in certain types of cancer (Kalender *et al*. 2010). A further independent AMPK effect is known to inhibit the serine-protein kinase ATM gene and reduce the level of ROS (Algire *et* al. 2012). The proposed pro-apoptotic effects of AMPK are produced by the mitochondrial complex-1 (Figure 20), and can confer the risk of mutagenesis (Algire *et* al. 2012). The inhibition of the mitochondrial complex 1 is

known to reduce the production of ROS, oxidative stress, DNA damage and thusly reducing the risk of mutagenesis (Algire *et al*. 2012).

Within cancer, Metformin is described to lower systemic glucose and insulin levels, thusly decreasing insulin-mediated tumour growth and progression, whilst Metformin's anti-inflammatory effects is said to potentially reduce the risk of developing cancer (Pollak *et al*. 2012, Pernicova and Korbonits 2014). From Figure 20, activation of AMPK is known to activate the tumour suppressor gene TSC-2, which inhibits the mTORC1 activator RHEB, AMPK further inhibits and directly phosphorylates a member of the mTORC1 complex referred to as Raptor (Gwinn *et al*. 2008). The lowering of insulin levels via AMPK-dependent phosphorylation of IRS-1 is hypothesised to decrease the downstream signalling of IGF–1-insulin receptors, therefore inhibiting Akt and mTORC1 signalling (Gunton *et* al. 2003, Ning and Clemmons 2010, Pernicova and Korbonits 2014).

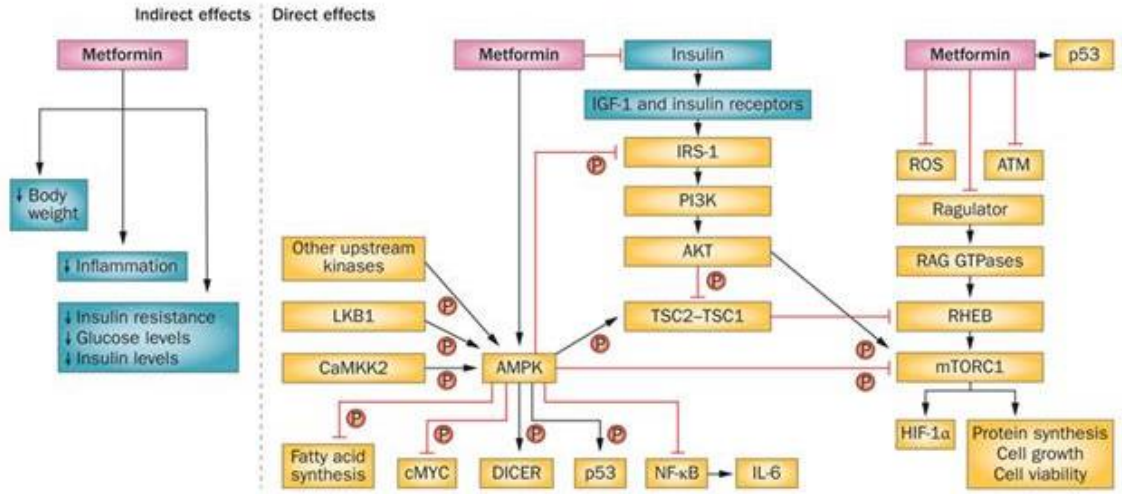

Figure 20: The anticancer effects of Metformin are hypothesised to result from the combination of indirect (systemic) and direct effects. As summarised by Pernicova and Korbonits (2014) Metformin's systemic influence can be demonstrated on metabolism and insulin – sensitive target tissues, whilst Metformin is known to lower system glucose and insulin levels, the decrease can result in the decrease in insulin–mediated tumour growth and progression. Metformin's direct effects can stem from the ability to inhibit the mTORC1; the inhibition of mTORC1 is hypothesised to result in a reduction in cancer risk. Within Metformin–treated cancer cells, the activation of AMPK can act to restrain cancer cell growth; the resulting inhibition of TSC-2 inhibits the mTORC1 activator RHEB as well as AMPK directly inhibiting the member of mTORC1 protein Raptor. Moreover, the ability by Metformin to decrease signalling downstream of IGF-1–insulin receptors results in the inhibition of the Akt and mTORC1 signalling pathways (Pernicova and Korbonits 2014).

The focus on Metformin's anticancer ability still remains divided as Metformin was shown to inhibit oxidative glycolysis in pre-neoplastic cells with an intact AMPK axis (Huang *et al*. 2008), however LKB1 and AMPK have been suggested to offer advantageous effects to tumour cells, in established tumours, protecting the cells against energetic stress (Jeon *et al*. 2012). The absence of either LKB1 or AMPK in established tumours was shown to result in a greater susceptibility to depleted ATP via Metformin, resulting from the impaired ability to restore the energy balance

(Algire *et al*. 2011). In acute myeloid leukaemia the LKB1-AMPK axis remains functional, however AMPK activation with Metformin demonstrated a full inhibition of mTORC1 activity, thereby reducing oncogenic protein synthesis (Green e*t al*. 2011). Incubation of leukaemia cancer cells with Metformin resulted in apoptotic cell death, whilst Metformin exerted significant anti-leukaemia activity in *in-vivo* mice transplanted with a human acute myeloid leukaemia cell line, highlighting the role of AMPK agonism for therapy (Green *et al*. 2010). From this, it would be interesting to see the potential anti-proliferative properties of Metformin during co-administration with a chemotherapeutic agent such as Sunitinib in leukaemia cells.

As discussed, Metformin has the ability to result in cancer cell death, we aim to investigate the potential for Metformin to be used in adjunctive treatment. Following this, we aim to investigate the anticancer properties of Metformin in Sunitinib-induced cytotoxicity with the potential for Metformin to be used in combination with Sunitinib in chemotherapy treatment. Using information discussed, it can be suggested that Metformin has the potential to provide an anti-neoplastic effect alone but also in combination with existing chemotherapeutic agents, such as Sunitinib. However, the idea is not novel, as multiple studies have considered the anti-neoplastic potential of Metformin when used in combination with cytotoxic therapies (Chen *et al*. 2012).

Metformin has been shown to enhance the anticancer effect of chemotherapeutic agents (Liu *et al*. 2012, Shi *et al*. 2012, Chen *et al*. 2012, Nilsson e*t al*, 2011, Hirsch *et al*. 2009, Erices *et al*. 2013). However, Metformin was able to antagonise Cisplatin-induced cytotoxicity in glioma, neuroblastoma, fibrosarcoma and leukaemia cell lines, believed to be via an AMPK-independent activation of Akt (Janjetovic *et al*. 2011). The use of Metformin with Sunitinib co-treatment in diabetic patients with renal cell carcinoma indicated that patients receiving Metformin had a significantly higher 11-month increase in overall survival, following adjustments for known risk factors for poorer outcomes (Keizman *et al*. 2016). It was said that patients had an increase in clinical benefit and a decrease in primary treatment refractions, however progression-free survival was not deemed statistically significant against non-Metformin treated patients (Keizman *et al*. 2016). Moreover, despite mentioning potential pathways associated with the stated outcomes the authors were not able to perform any analysis in regards to evaluating the cellular

and anticancer hypotheses. Furthermore, the authors highlighted that the study performed was carried out with only 52 Metformin users and 56 non-Metformin users, whilst the patients only represented a heterogeneous group of patients and the study did not take into consideration the duration of Metformin therapy during Sunitinib treatment in patients (Keizman e*t al*. 2016). The authors further stated that the significant decrease of LVEF, acute coronary syndrome and cerebrovascular accident during therapy was said to be similar between the groups of patients (Keizman *et al*. 2016), this would contradict existing reports highlighting Sunitinib's ability to reduce LVEF as well as other cardiovascular events. It is worth addressing these issues when investigating the potential use of Metformin cotreatment with Sunitinib.

In-vivo and *in-vitro* studies have demonstrated that Metformin may increase the efficacy of standard anticancer drugs (Vujic *et al*. 2015, Jang *et al*. 2014, Sliwinska *et al*. 2015). Metformin co-treatment was suggested to allow for chemotherapeutic dosage to be decreased without reducing effectiveness (Chen e*t al*. 2015). Metformin was shown to accelerate WP 631-induced cell death, whilst enabling a dose reduction of WP 631 without the loss of the growth-inhibition effect on HepG2 cells (Sliwinska e*t al*. 2015). Sliwinska e*t al*. (2015) commented that these results were in accordance with that shown by Chen *et al*. (2015) and Illiopoulos *et al*. (2011). The decrease in p53 levels in HepG2 cells, following WP 631 co-treatment with Metformin, was said to be the result of the activation of AMPK and NAD(+)-dependent histone/protein deacetylase sirtuin 1 (SIRT1) (Nelson *et al*. 2012, Sliwinska e*t al*. 2015). Nelson *et al*. (2012) indicated that Metformin increased susceptibility of p53 to ubiquitination by MDM2 via the activation of AMPK-SIRT1 pathway, promoting the deacetylation of p53 (Sliwinska *et al*. 2015). HepG2 cells are said to be resistant to chemotherapeutic cytotoxic agents as a result of PTEN inactivation and Akt hyperactivity, whilst also possessing hyperactive Akt, which is said to be involved in the regulation of NFκB (Sliwinska *et al*. 2015). NFκB is responsible for the regulation of the genetic expression of cytokines, chemokines, growth factors, cell surface receptors, enzymes and acute phase proteins (Wardle 2001, Rogalska *et al*. 2016). The NFκB signalling pathway is associated with cell survival, whilst affecting the course of pathways in cancer cells via the transcription of specific genes encoding proteins for the regulation of the cell cycle (cyclin D1,

growth arrest and DNA damage–inducible 45 (GADD45) and the tumour suppressor gene p161^{INK4A}), apoptosis B cell lymphoma–XL (BclXL), x–linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis (cIAP) and oncogenesis (p53, vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM) (Rogalska *et al*. 2016). However, reports indicate that NFκB is involved in both apoptotic and anti-apoptotic properties (Rogalska *et al*. 2016, Ueda and Richmond 2006, Aoki *et al*. 2001, Ismail *et al*. 2015).

AMPK is a known sensor of cellular energy status and activation results in the switching on of catabolic pathways that generate ATP and switches off ATP-dependent processes (Hardie 2004, Hasinoff *et al*. 2008). With this, AMPK is a critical regulator of energy homeostasis in metabolic processes and inhibits the rate-limiting steps in lipogenesis, including SREBP-1 via mTOR in hepatic cells, repression of AMPK is suggested to negate the mentioned effects (Scott *et al*. 2004, Lv *et al*. 2015). As we've outlined previously, Metformin could improve hepatic steatosis by increasing AMPK phosphorylation (Hu *et al*. 2013, Lv *et al*. 2015). Lv *et al*. (2015) demonstrated that activation of AMPK was able to reverse the effects of TNF-α on lipid disposition in HepG2 cells. This supported the suggestion that TNF-α induced skeletal muscle insulin resistance is involved in AMPK signalling suppression (Lv *et al*. 2015). HepG2 cells are said to exhibit chromosomal and oncogenic gene abnormalities, as well as expressing wild-type p53 (Nelson e*t al*. 2012). Using this, the study by Nelson *et al*. (2012) demonstrated that addition of 2 mM Metformin was able to decrease p53 protein concentration, under high glucose conditions. Furthermore, addition of Metformin in HepG2 cells was able to diminish systolic reactive oxygen species production in high glucose conditions (Nelson *et al*. 2012). The findings were said to be similar to studies involving AMPK activation in glomerular epithelial cells (Nelson *et al*. 2012, Eid *et al*. 2010).

In-vitro studies for human myeloid leukaemia can be carried out involving the HL60 cell line, originating from a female patient with acute myeloid leukaemia, an attractive model for studying cell differentiation and human myeloid cell differentiation (Birnie 1988). As outlined previously, we aim to investigate the potential role of Metformin in Sunitinib-induced cytotoxicity using the HL60 cell line. Based on knowledge involving Metformin treatment of HepG2 cells, we further aim

to investigate the role of Metformin with Sunitinib using the HepG2 cell line, providing a comparison to the potential use of Metformin together with Sunitinib.

Using this information, we propose that Metformin has the potential to be used in co-administration with Sunitinib treatment. We have previously demonstrated in Chapters 3-4, that Metformin has the ability to reduce Sunitinib–induced cardiac myocyte death as well as reduce Sunitinib–induced increase in infarct percentage of the myocardium. However, with this we need to investigate whether Metformin is able to be used in combination with Sunitinib treatment, and if Metformin will result in a synergistic effect during co–administration in cancer cell lines. Moreover, we aim to demonstrate the potential role of AMPK signalling pathway during Metformin co–administration with Sunitinib. In doing so, we aim to use the MTT assay. HepG2 and HL60 cells will be used to assess cell metabolic activity including NADPH–dependent cellular oxidoreductase enzymes to reduce tetrazolium dye MTT to the insoluble formazan product.

5.2. Methodology.

5.2.1. Chemicals.

Metformin hydrochloride (500 mg, MW 165.62 mg/mM) was purchased from Sigma Aldrich and dissolved in ultra-pure RO water. The Metformin stock solution was stored at -20^{o}C.

Sunitinib malate (25 mg, MW 532.56 mg/mM) was purchased from Sigma Aldrich and dissolved in DMSO. The Sunitinib stock solution was stored at -20^{o}C and care was taken to avoid exposure to light and UV radiation.

S-(4-Nitrobenzyl)-6-thioinosine (NBTI), 100 mg, MW 419.41 mg/mM) was purchased from Sigma Aldrich and was dissolved in DMSO. The NBTI stock solution was stored at -20^{o}C.

HepG2 and HL60 cancer cell lines were acquired from ATCC brought up from the Coventry University reserved stock.

Penicillin-Streptomycin Solution (x100 concentration), Trypsin with EDTA (x10 concentration) and L-Glutamine (200 mM, x100 concentration) were all purchased from LabTech International Ltd, East Sussex, United Kingdom. FBS, RPMI 1640 Medium and DMEM with high glucose, L-Glutamine and Sodium Pyruvate w/o HEPES were all purchased from Thermo Fisher Life Technologies Ltd, Paisley, United Kingdom. Remaining reagents were standard laboratory reagents from Sigma Aldrich and Fisher Scientific.

5.2.2. MTT Assay for HepG2 and HL60 Cell Viability.

As mentioned in Section 2.5., the MTT assay with 3-(-4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide was performed using HepG2 and HL60 cancer cells for control and treatment groups (Section 2.5.). Cells were incubated with (i) Metformin (6-1000 µM), (ii) Sunitinib (0.1-100 µM), (iii) the combination (co–treatment) of Sunitinib (0.1-100 µM) and Metformin (50 µM), (iv) NBTI (0.1–100 µM), (v) the combination (co–treatment and NBTI) of Sunitinib (0.1-100 µM) and Metformin (50 µM) and NBTI (1 µM).

HepG2 and HL60 cells were investigated for assessing cell metabolic activity including NADPH–dependent cellular oxidoreductase enzymes to reduce tetrazolium dye MTT to the insoluble formazan product.

HepG2 cells were plated and incubated at 37ºC, 5 % CO_2 for 48 hours, to allow attachment and 40%-60% confluency. Plates containing HL60 cells were incubated at 37ºC, 5 % CO_2 for 24 hours, to allow attachment and 40-60 % confluency

Plates were incubated with required drug concentration for 24 hours. Following incubation with compounds, media was replaced with MTT media (concentration 5 mg/ml). Plates were incubated for 6 hours minimum at 37ºC, 5 % CO_2. MTT-containing media was removed gently and replaced with DMSO. Plates were read on microtiter plate reader at 595 nm/492 nm, reference 690 nm. Treatment groups were standardised to vehicle control for each plate. EC_{50} was generated from the dose-response curve for each sample using GraphPad PRISM software.

5.2.3. Statistical Analysis.

The data were expressed as mean ± SEM of 4-6 wells for each treatment group and concentration. Sunitinib doses, the combination of Metformin and Sunitinib, and the combination of Metformin, Sunitinib and NBTI were assessed for the EC_{50} and statistical difference ($p<0.05$) using one-way ANOVA with LSD post hoc tests by GraphPad PRISM for each treatment dosage and overall mean value.

5.3.1. Results.

Results from 90 successful experiments, consisting of 3 plates per replicate (n) for 6 replicates, were included for the MTT assay study to assess the effect on MTT reductase activity levels during Sunitinib treatment at doses 0.1–100 μM (n6) (Figures 21a-21b), Metformin treatment at doses 6–1000 μM (n6) (Figures 22a–22b), the combination (co–treatment) of Sunitinib at doses 0.1–100 μM and Metformin 50 μM (n6) (Figures 23a–23b), NBTI at doses 0.1–100 μM (n6) (Figures 24a–24b) and the combination (co–treatment and NBTI) of Sunitinib at doses 0.1–100 μM and Metformin 50 μM and NBTI 1 μM (n6) (Figures 25a–25b).

5.3.2. The effects of Sunitinib on cell viability of HepG2 cells.

Figure 21a the effects of Sunitinib treatment on cell viability of HepG2 cells

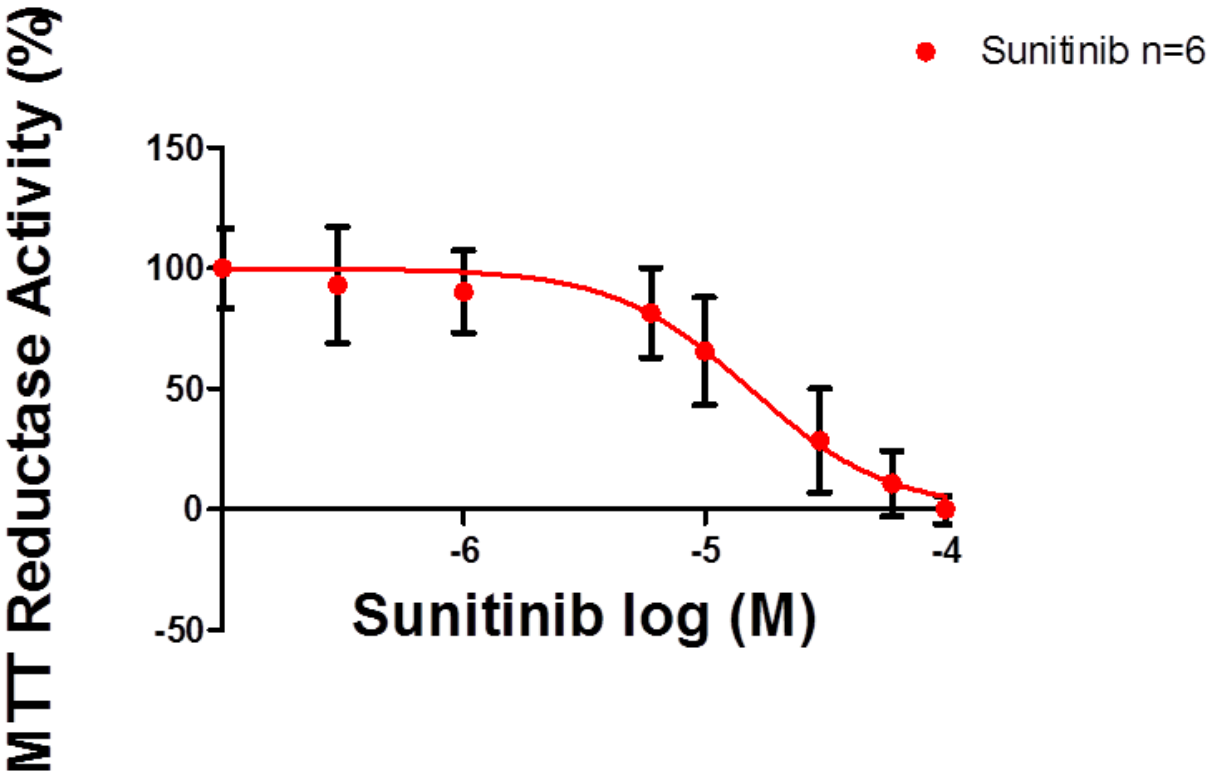

Figure 21a: MTT assay for Sunitinib treatment dose–response on HepG2 cells following 24 hours of incubation using concentrations 0.1–100 µM (n=6). Treatment groups were standardised to control group for each individual plate.

From Figure 21a Sunitinib at concentrations 1–100 µM demonstrated a significant decrease in cell viability of HepG2 cells compared to the control ($p<0.05$, EC_{50} 15.4 µM). Cell viability was demonstrated to be 88 ± 7 % following incubation with Sunitinib at 1 µM concentration, moreover cell viability was demonstrated to be 11 ± 2 % following incubation with Sunitinib at 100 µM concentration.

5.3.3. The effects of Sunitinib on cell viability of HL60 cells.

Figure 21b the effects of Sunitinib treatment on cell viability of HL60 cells

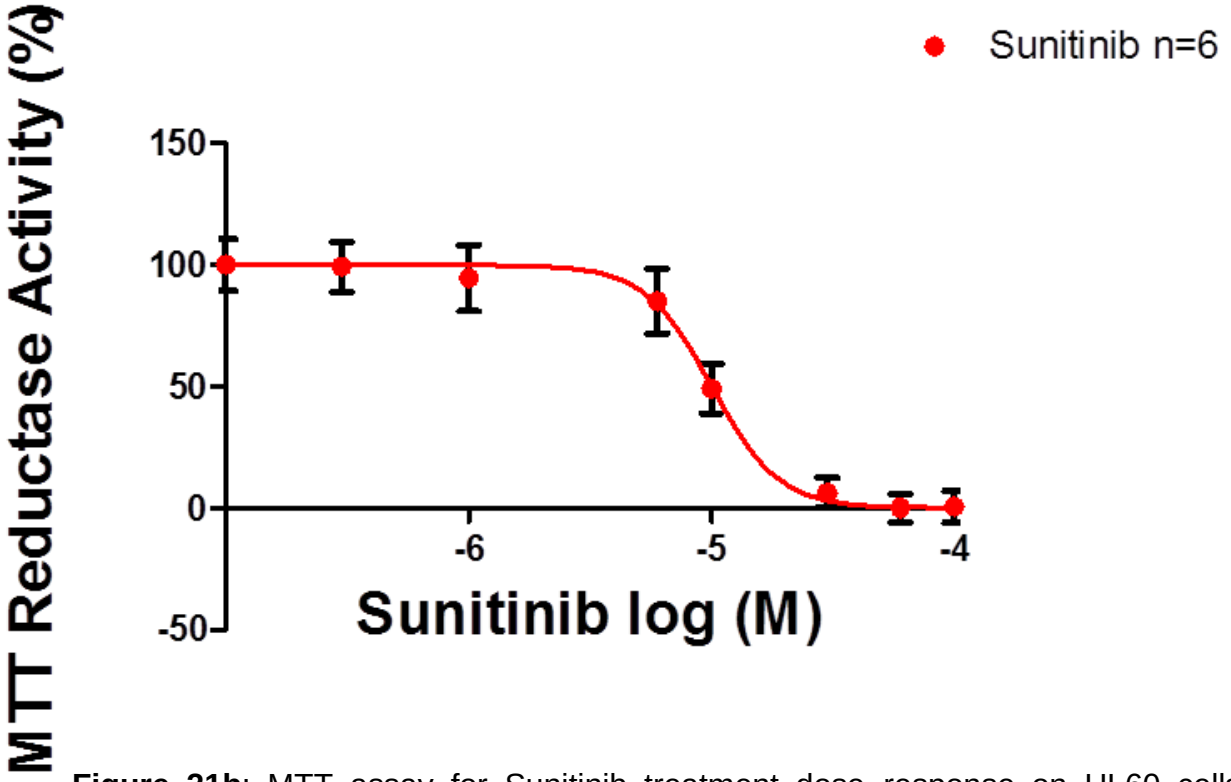

Figure 21b: MTT assay for Sunitinib treatment dose–response on HL60 cells following 24 hours of incubation using concentrations 0.1–100 µM (n=6). Treatment groups were standardised to control group for each individual plate.

From Figure 21b Sunitinib at concentrations 0.1–100 µM demonstrated a significant decrease in cell viability of HL60 cells compared to the control ($p<0.05$, EC_{50} 10 µM). Cell viability was demonstrated to be 86 ± 5 % following incubation with Sunitinib at 1 µM concentration, moreover cell viability was demonstrated to be 15 ± 2 % following incubation with Sunitinib at 100 µM concentration.

5.3.4. The effects of Metformin on cell viability of HepG2 cells.

Figure 22a the effects of Metformin treatment on cell viability of HepG2 cells

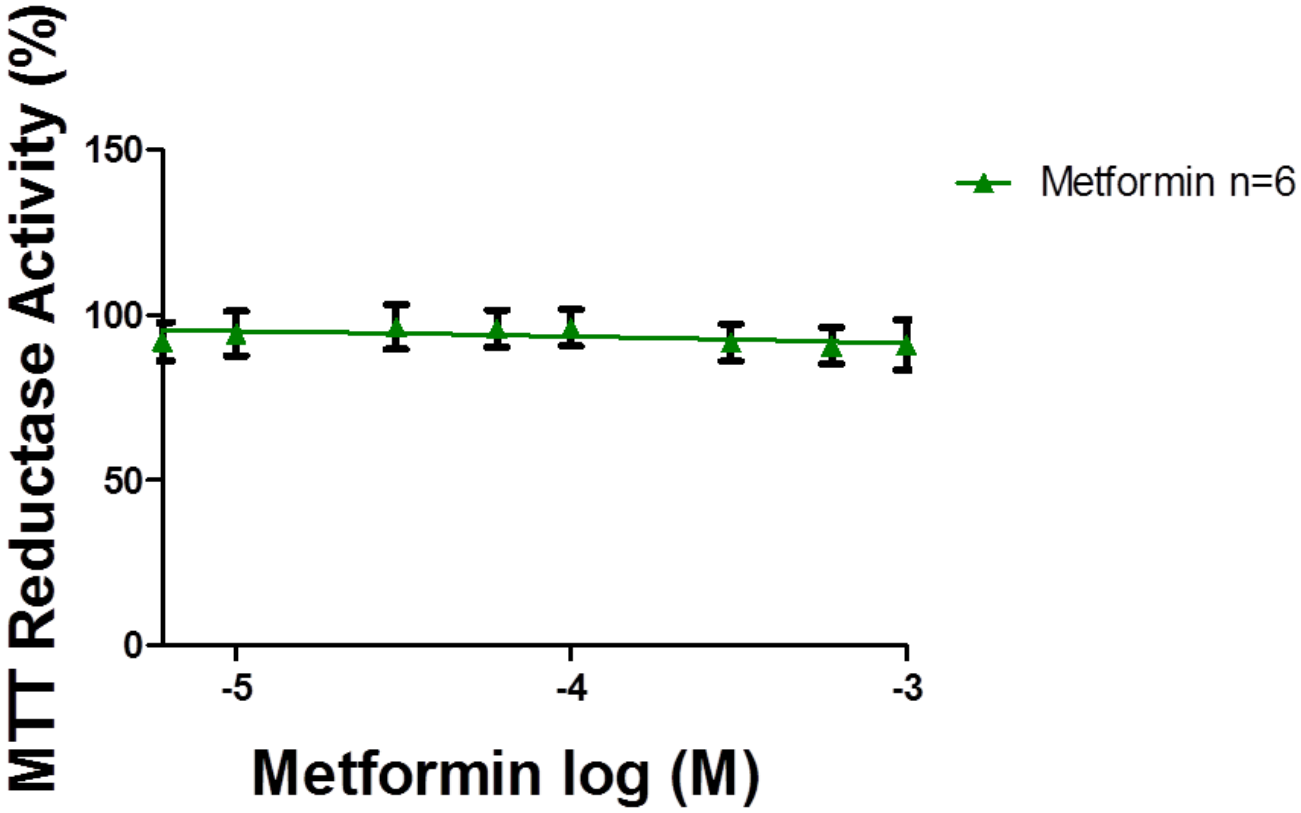

Figure 22a: MTT assay for Metformin treatment dose–response on HepG2 cells following 24 hours of incubation using concentrations 6–1000 µM (n=6). Treatment groups were standardised to control group for each individual plate.

From Figure 22a Metformin at concentrations 6-1000 µM demonstrated no significant decrease in cell viability in HepG2 cells compared to the control group, below 100 % cell viability, following standardisation to each control ($p>0.05$).

5.3.5. The effects of Metformin on cell viability of HL60 cells.

Figure 22b the effects of Metformin treatment on cell viability of HL60 cells

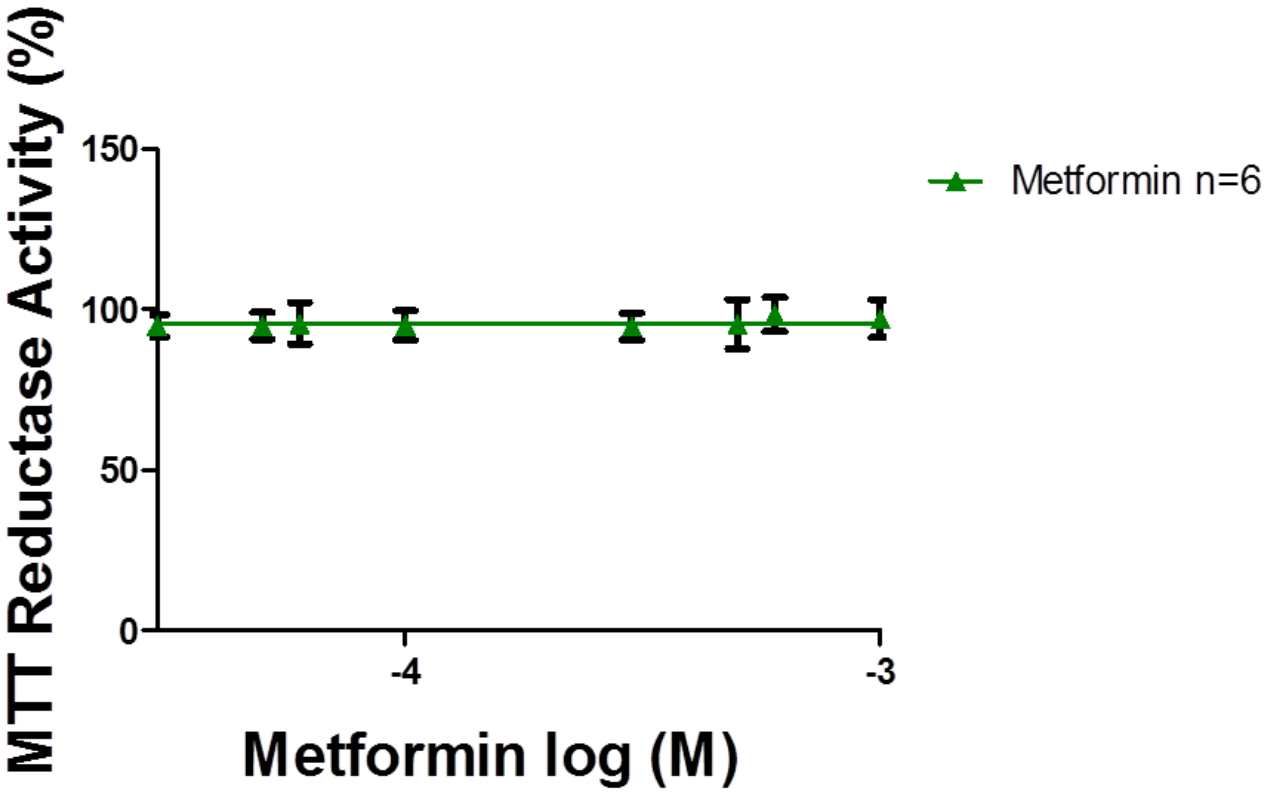

Figure 22b: MTT assay for Metformin treatment dose–response on HL60 cells following 24 hours of incubation using concentrations 30–1000 µM (n=6). Treatment groups were standardised to control group for each individual plate.

From Figure 22b Metformin at concentrations 30-1000 µM demonstrated no significant decrease in cell viability of HL60 cells compared to the control group, below 100 % cell viability, following standardisation to each control ($p>0.05$).

5.3.6. The effects of Sunitinib in the absence and presence of Metformin on cell viability of HepG2 cells

Figure 23a the effects of Sunitinib in the absence and presence of Metformin treatment on cell viability of HepG2 cells

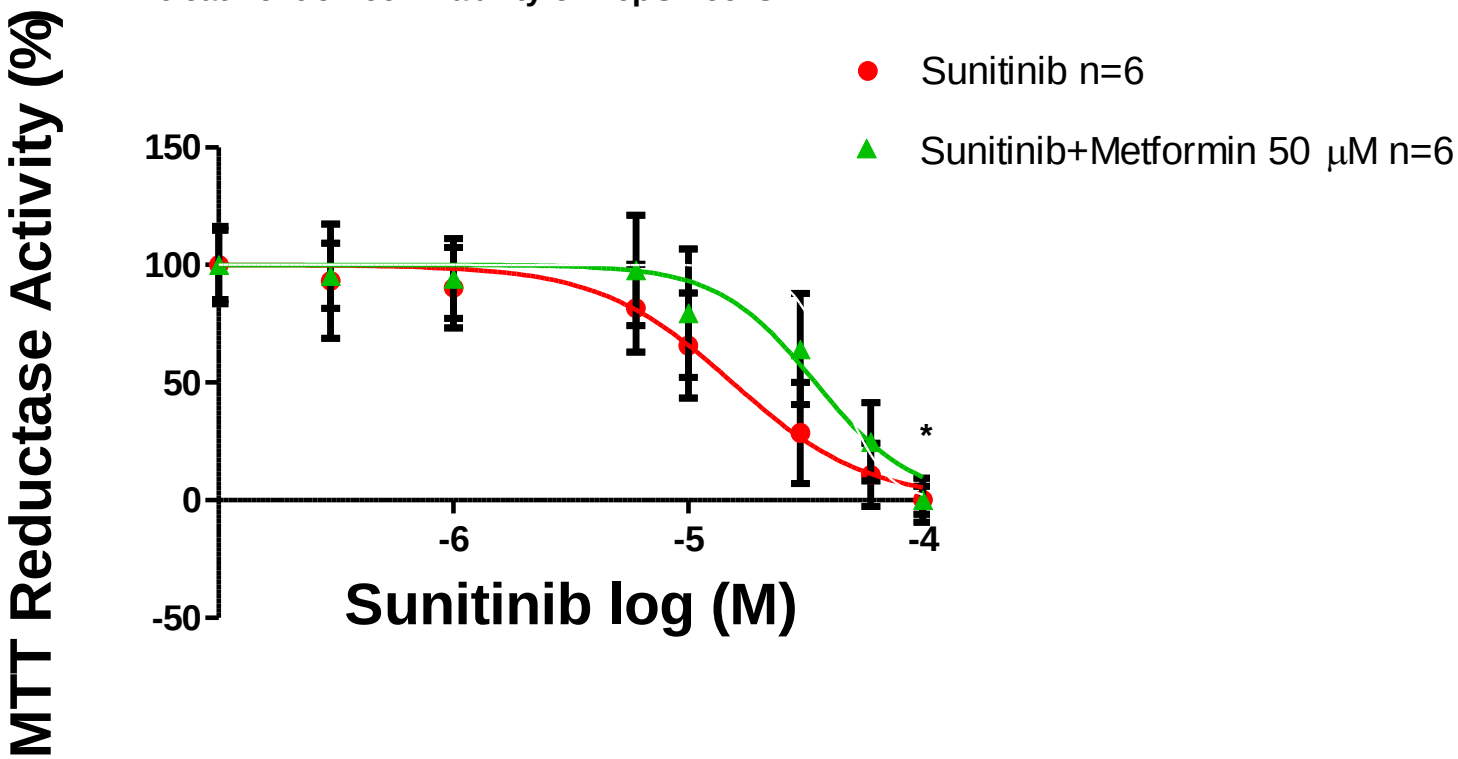

Figure 23a: MTT assay for Sunitinib treatment dose–response on HepG2 cells following 24 hours of incubation using concentrations 0.1–100 µM (n=6) in the absence and presence of Metformin (50 µM) (n=6). Treatment groups were standardised to control group for each individual plate. *Key: *=p<0.05 Co-treatment vs Sunitinib.*

From figure 23a the combination of Sunitinib at concentrations 0.1–100 µM and Metformin at 50 µM (co-treatment) demonstrated an overall significant increase in cell viability of HepG2 cells compared to the Sunitinib at concentrations 0.1–100 µM alone ($p<0.05$, EC_{50} 34.7 µM for co–treatment vs. EC_{50} 15.4 µM for Sunitinib). Figure 23a demonstrated a right–shift of the curve for the Sunitinib and Metformin co–treatment group when compared to the Sunitinib–treatment group alone. The co-treatment of 50 µM Metformin with Sunitinib at 100 µM concentration demonstrated a significant increase in cell viability at compared to Sunitinib at 100 µM alone ($p<0.05$, 18 ± 3 % vs. 11 ± 2 %).

5.3.7. The effects of Sunitinib in the absence and presence of Metformin on cell viability of HL60 cells

Figure 23b the effects of Sunitinib in the absence and presence of Metformin treatment on cell viability of HL60 cells

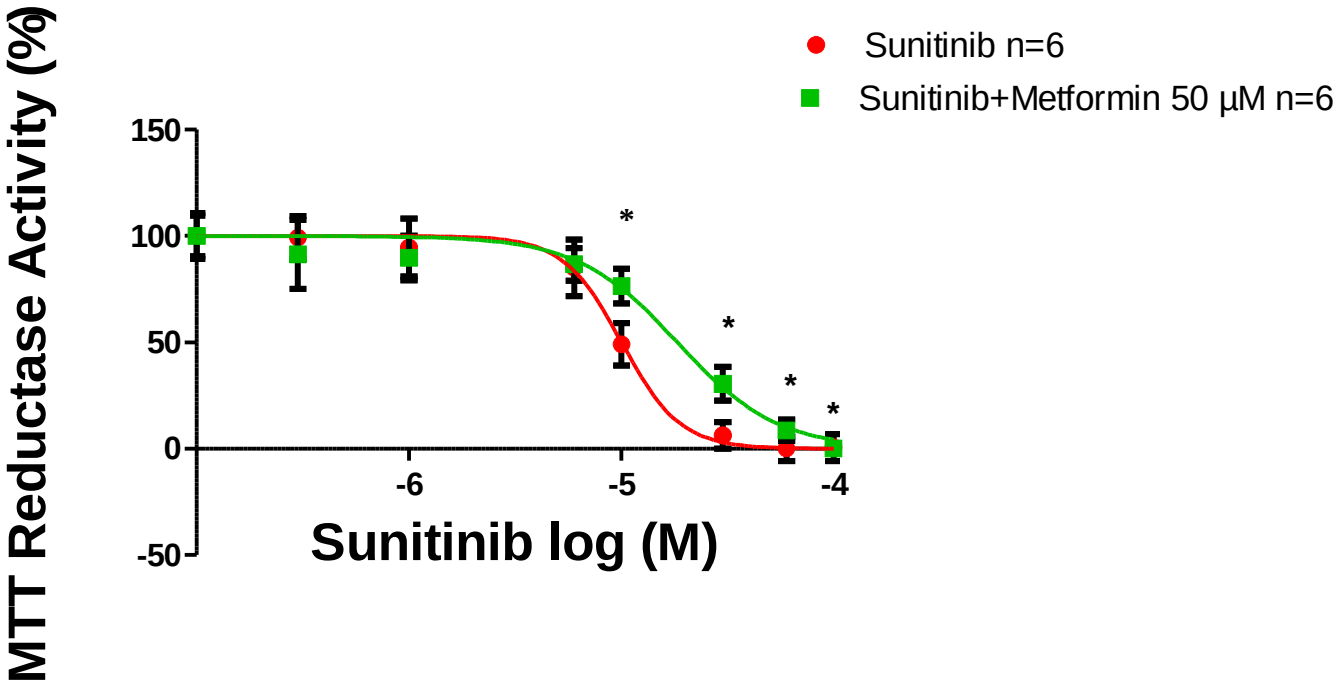

Figure 23b: MTT assay for Sunitinib treatment dose–response on HL60 cells following 24 hours of incubation using concentrations 0.1–100µM (n=6) in the absence and presence of Metformin (50 µM) (n=6). Treatment groups were standardised to control group for each individual plate. *Key: *=p<0.05 Co-treatment vs Sunitinib.*

From Figure 23b the combination of Sunitinib at concentrations 0.1–100 µM and Metformin at 50 µM (co-treatment) demonstrated an overall significant increase in cell viability of HL60 cells compared to the Sunitinib at concentrations 0.1–100 µM alone ($p<0.05$, EC_{50} 18.2 µM for co–treatment vs. 10 µM for Sunitinib). Figure 23b demonstrated a right–shift of the curve for the Sunitinib and Metformin co–treatment group when compared to the Sunitinib–treatment group alone. Co-treatment of Sunitinib at 100 µM concentration with 50 µM Metformin demonstrated a significant increase in cell viability compared to Sunitinib at 100 µM alone ($p<0.05$, 19 ± 2 % vs. 15 ± 2 %. Furthermore, Sunitinib at concentrations 30, 60 and 100 µM with 50 µM Metformin demonstrated a significant increase in cell viability when compared

to Sunitinib 10μM (77 ± 3 % vs. 52 ± 3 %), 30 μM (46 ± 4 % vs. 20 ± 2 %) and 60 μM (30 ± 5 % vs. 15 ± 2 %) treatment alone.

5.3.8. The effects of NBTI on cell viability of HepG2 cells.

Figure 24a the effects of NBTI treatment on cell viability of HepG2 cells

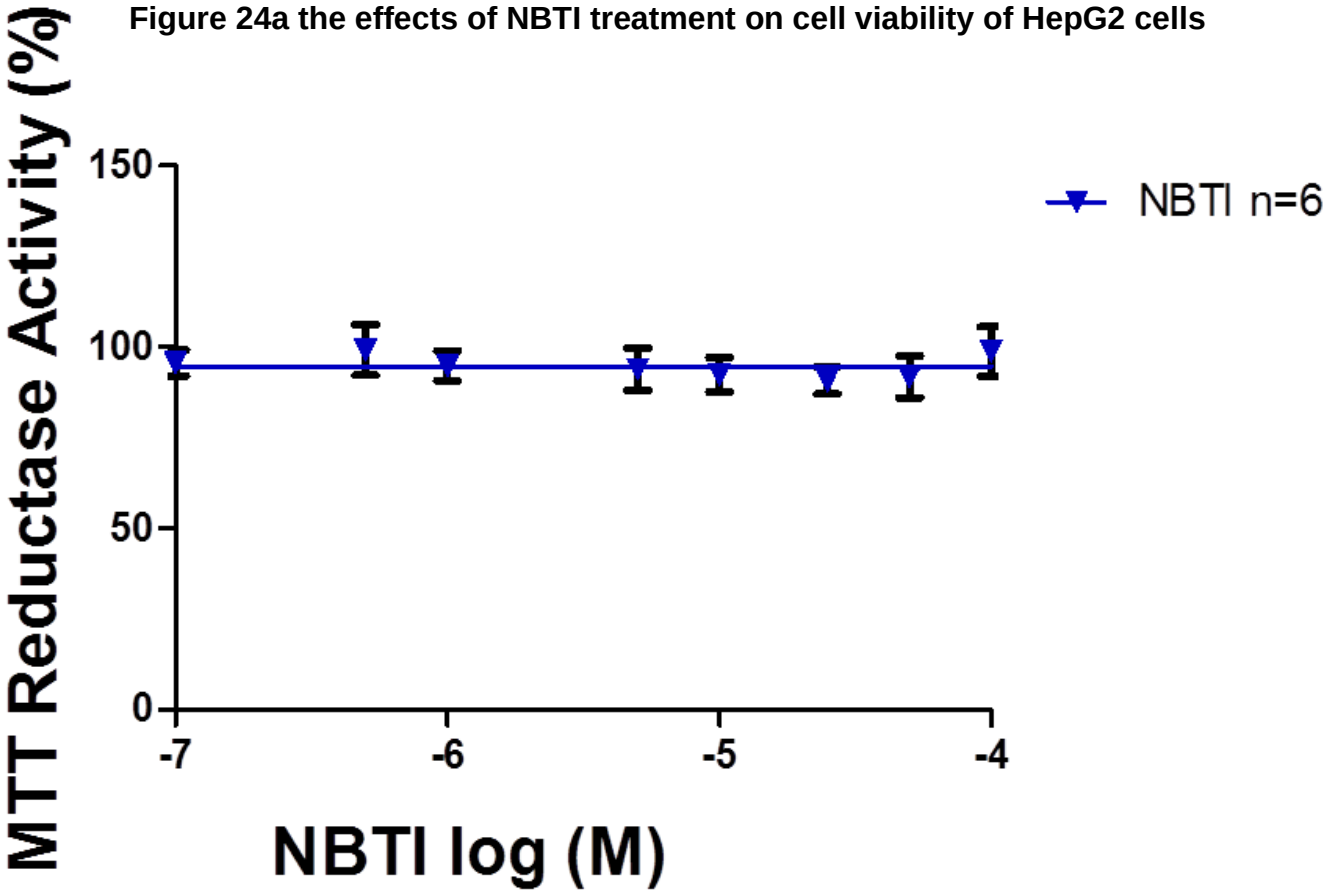

Figure 24a: MTT assay for NBTI treatment dose–response on HepG2 cells following 24 hours of incubation using concentrations 0.1–100 µM (n=6). Treatment groups were standardised to control group for each individual plate.

From Figure 24a it was demonstrated that NBTI administration alone at concentrations 0.1–100 µM did not result in a significant decrease in cell viability on HepG2 cells.

5.3.9. The effects of NBTI on cell viability of HL60 cells.

Figure 24b the effects of NBTI treatment on cell viability of HL60 cells

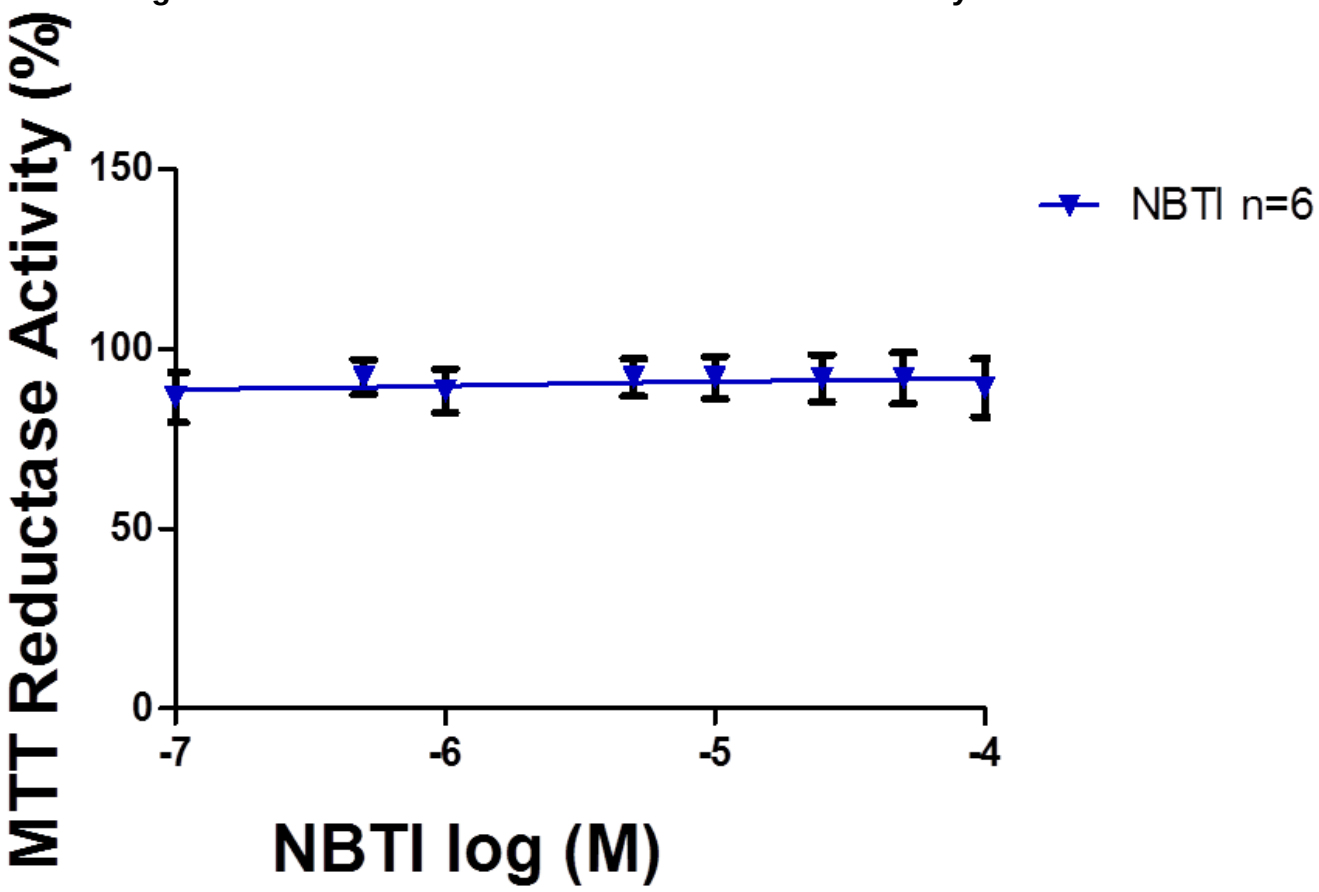

Figure 24b: MTT assay for NBTI treatment dose–response on HL60 cells following 24 hours of incubation using concentrations 0.1–100 µM (n=6). Treatment groups were standardised to control group for each individual plate.

From Figure 24b it was demonstrated that NBTI administration alone at concentrations 0.1–100 µM did not result in a significant effect in cell viability on Hl60 cells.

5.3.10. The effects of Sunitinib in the absence and presence of Metformin and NBTI on cell viability of HepG2 cells

Figure 25a the effects of Sunitinib in the absence and presence of Metformin and NBTI treatment on cell viability of HepG2 cells

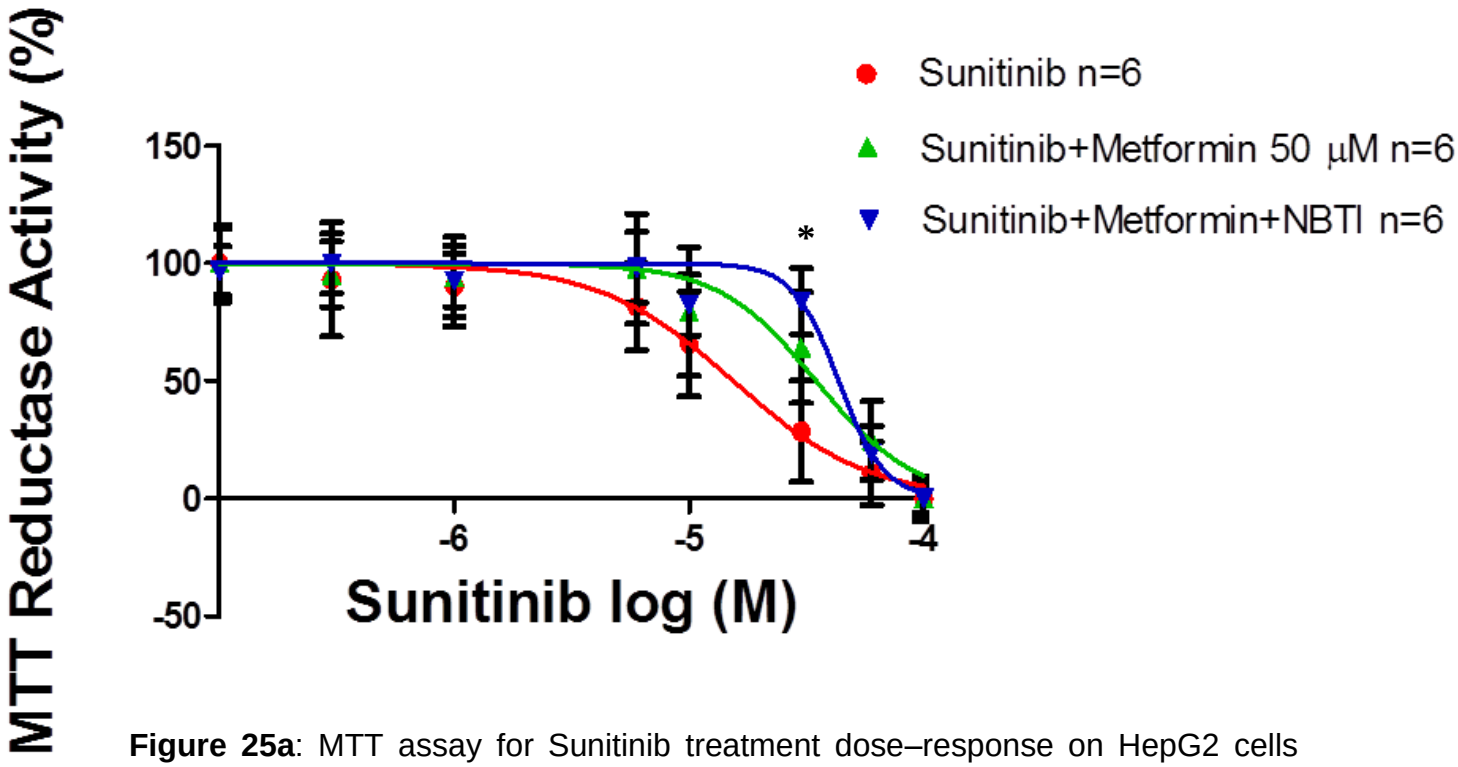

Figure 25a: MTT assay for Sunitinib treatment dose–response on HepG2 cells following 24 hours of incubation using concentrations 0.1–100 µM (n=6) in the absence and presence of Metformin (50 µM) (n=6) and NBTI (1 µM) (n=6). Treatment groups were standardised to control group for each individual plate. *Key: *=p<0.05 Co–treatment+NBTI vs. Sunitinib.*

From Figure 25a the combination of Sunitinib at concentrations 0.1–100 µM, Metformin at 50 µM and NBTI 1 µM (co-treatment and NBTI) demonstrated an overall significant increase in cell viability of HepG2 cells compared to Sunitinib at concentrations 0.1–100 µM alone ($p<0.05$, EC_{50} 43.3 µM for co–treatment and NBTI vs. 15.4 µM for Sunitinib) and an overall significant increase in cell viability when compared to the combination of Sunitinib and 50 µM Metformin (co–treatment) group alone ($p<0.05$, EC_{50} 43.3 µM for co–treatment and NBTI vs. 34.7 µM for co-treatment). Figure 25a demonstrated a right–shift of the curve for the co–treatment and NBTI group when compared to the co–treatment group and when compared to the Sunitinib treatment group. The combination of Sunitinib, Metformin 50 µM and

NBTI 1 µM demonstrated a significant increase in cell viability when compared to the Sunitinib treatment group at 30 µM concentration group ($p<0.05$, 89 ± 6 % vs. 35 ± 8 %).

5.3.11. The effects of Sunitinib in the absence and presence of Metformin and NBTI on cell viability of HL60 cells

Figure 25b the effects of Sunitinib in the absence and presence of Metformin and NBTI treatment on cell viability of HL60 cells

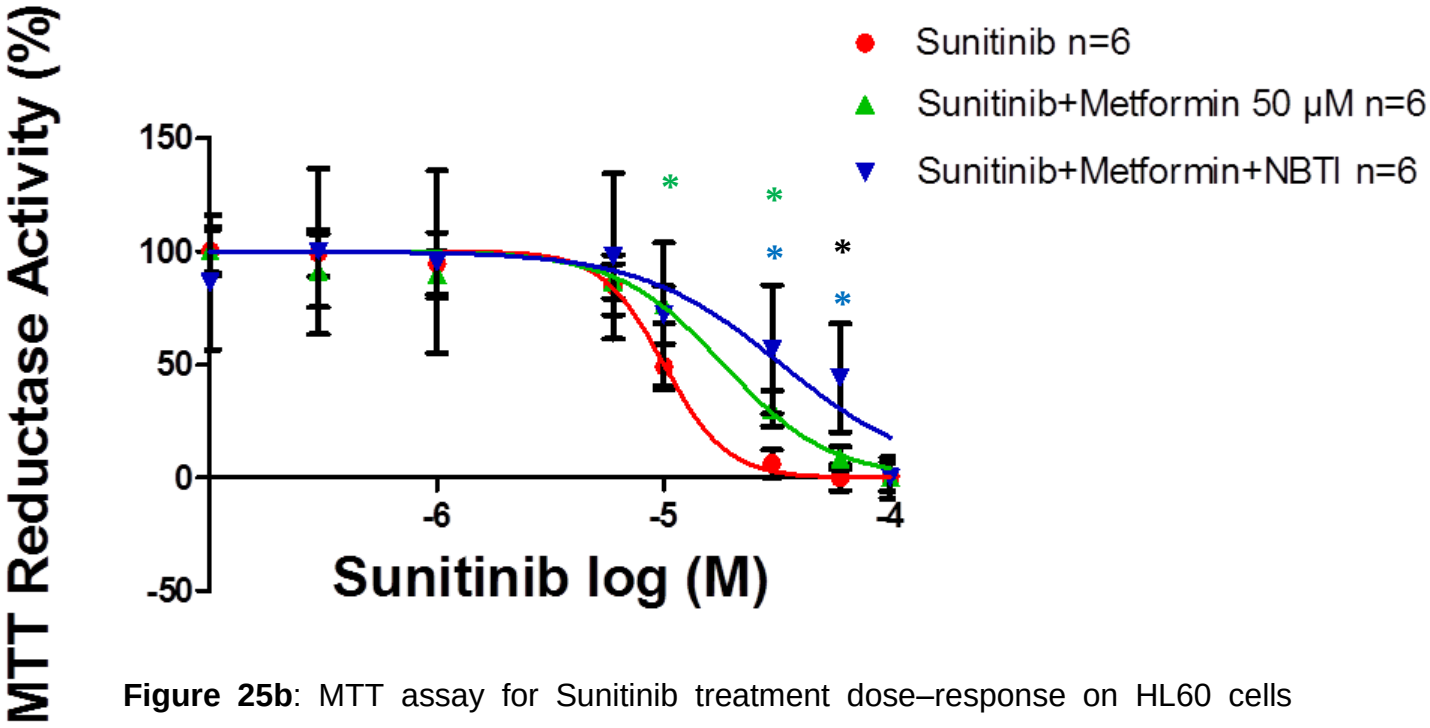

Figure 25b: MTT assay for Sunitinib treatment dose–response on HL60 cells following 24 hours of incubation using concentrations 0.1–100 µM (n=6) in the absence and presence of Metformin (50 µM) (n=6) and NBTI (1 µM) (n=6). Treatment groups were standardised to control group for each individual plate. *Key: *=p<0.05 Co–treatment+NBTI vs. Sunitinib. *=p<0.05 Co–treatment vs. Sunitinib. *=p<0.05 Co–treatment+NBTI vs. Co-treatment.*

From Figure 25b the combination of Sunitinib at concentrations 0.1–100 µM, Metformin at 50 µM and NBTI 1 µM (co-treatment and NBTI) demonstrated an overall significant increase in cell viability of HL60 cells compared to the Sunitinib at concentrations 0.1–100 µM alone ($p<0.05$, EC_{50} 33 µM for co–treatment and NBTI vs. 10 µm for Sunitinib), and an overall significant increase in cell viability when compared to the Sunitinib and Metformin 50 µM (co–treatment) group ($p<0.05$, EC_{50} 33.3 µM for co–treatment and NBTI vs. 18.3 µM for co-treatment). Figure 25b demonstrated a right–shift of the curve for the co–treatment and NBTI group when compared to the co–treatment group and when compared to the Sunitinib treatment

group. The combination of Sunitinib, Metformin 50 µM and NBTI 1 µM demonstrated a significant increase in cell viability compared to the co-treatment of Metformin 50µM and Sunitinib at 60µM concentration ($p<0.05$, 59 ± 8 % vs. 30 ± 5 %). Moreover, the combination of Sunitinib, 50 µM Metformin and NBTI 1µM demonstrated a significant increase in cell viability compared to the Sunitinib treatment group at 30 µM concentration ($p<0.05$, 69 ± 10 % vs. 20 ± 2 %) and at 60 µM Sunitinib concentration ($p<0.05$, 59 ± 8 % vs. 15 ± 2 %).

5.4. Discussion

During cancer treatment, when the cancer is said to be incurable the improvement in overall survival via treatment with a potentially cardiotoxic agent will outweigh the associated safety risk (Mellor *et al*. 2010). Moreover, if the drug treatment can be used as an adjunctive form, the balance between risk and benefit will need to be examined carefully. To date, the TKI Sunitinib is administered, despite reports of adverse cardiac events, due to the drug being highly effective within settings where treatment is limited especially during oncology drug development where there is an issue in achieving anticancer efficiency is considered to be great, the risk of cardiotoxicity is considered on balance (Mellor *et al*. 2010).

MTT assay results from Figures 21a and 21b demonstrated that Sunitinib resulted in a dose–dependent decrease in both HepG2 liver cancer cell viability and HL60 human leukaemia cell viability. It can be said that cell viability was shown to be inhibited at Sunitinib concentrations greater than 10 µM (Figures 21a–21b). To support this, it is known that Sunitinib is able to inhibit angiogenesis of cancer cells, as well as immune modulation and induce apoptosis in order to mediate an anti–tumour effect (Mena *et al*. 2010). Although the direct effect on tumour cells by Sunitinib is limited, using existing knowledge of chemotherapy-induced anti-tumour properties, it can be suggested that Sunitinib initiated a pro-apoptotic response involving mitochondria-mediated apoptosis (Yu and Zhang 2004). The proposed effect of Sunitinib can be deduced from Sunitinib's mode of action binding to overexpressed pro–angiogenic growth factors VEGFR and PDGFR, both required for tumour cell angiogenesis (Faivre *et al*. 2007). The ability for Sunitinib to bind to VEGFRs, stem–cell factors and PDGFRs was demonstrated to result in a pro-apoptotic response following the inhibition of the growth of human vein endothelial cells (Mendel *et al*. 2003). Sunitinib-induced activation of apoptosis in cancer cells was shown to be suppressed with an overexpression of the Bcl-2 homologue and anti-apoptotic protein ML1 myeloid cell leukaemia (MLC-1) (Sun *et al*. 2012, Fujise *et al*. 2000). In particular Sunitinib was demonstrated to activate PUMA expression in colon cancer cells, thusly resulting in the inhibition of Akt (Sun *et al*. 2012). PUMA is said to have an essential role in p53-dependent and independent apoptosis in human cancer cells, whilst also shown to activate mitochondrial-mediated apoptosis

via Bcl-2 family members Bax/Bak and suppressing anti-apoptotic Bcl-2 proteins (Sun *et al*. 2012, Yu *et al*. 2001, Yu *et al*. 2003, Ming *et al*. 2006). Moreover, DNA damage of tumour cells demonstrated p53-dependent induction of PUMA and apoptosis (Yu *et al*. 2003, Wang *et al*. 2007). Results from figures 21a and 21b can be suggested to be attributed to Sunitinib's involvement in the PI3K/Akt pathway; in particular the activation of an apoptotic response (Sun *et al*. 2012).

Previous studies demonstrated Sunitinib resulted in a concentration–dependent decrease in cell viability (Mendel *et al*. 2003). Results from the MTT assay dose-response demonstrated that Sunitinib treatment produced an EC_{50} concentration of 15.4 µM in HepG2 cells and 10 µM in HL60 cells in Figures 21a, 21b, 23a and 23b. The EC_{50} concentration demonstrated in our results is greater than the steady–state blood concentrations in patients undergoing Sunitinib treatment (Goodman *et al*. 2007, Henderson e*t al*. 2013). However, for Sunitinib to demonstrate a cytotoxic response, it was demonstrated in glucose and galactose-grown cancer cells that EC_{50} for Sunitinib was established to be 14 µM in glucose-grown cells and 14 µM in galactose-grown cells (Will *et al*. 2008), both concentrations were greater than the EC_{50} concentrations obtained in our study with HL60 cells but less than the EC_{50} value achieved with HepG2 cells. As well as a pro–apoptotic response, results in Figures 21a–21b can also suggest that Sunitinib may be potentially initiating in an inhibition of ATP or the limiting of ATP to cancer cells. TKIs are known to compete with ATP for binding to various wild–type and mutated receptor TKs (Faivre *et al*. 2007). The effect on ATP content following Sunitinib administration was demonstrated by Will *et al*. (2008) using the myoblast cell line H9c2, an *in-vitro* model to demonstrate cardiac related findings particularly direct mitochondrial functional impairment to cytotoxicity (L'Ecuyer e*t al*. 2001, Sardao e*t al*. 2008). Similar to our data in Figures 21a and 21b, the data generated by Will e*t al*. (2008) suggests that a concentration greater than the clinically relevant dose of 1 µM is required to achieve cell death, whilst a concentration greater than 10 µM is needed to achieve 100 % inhibition of ATP content and conclude cell death. Sunitinib was also said to inhibit oxidative phosphorylation complexes at concentrations above clinical C_{max} values (Will e*t al*. 2008).

Moreover, we have previously demonstrated that Sunitinib treatment at 1 µM using the Langendorff system was able to result in a dephosphorylation of p-AMPK signalling in animal cardiac tissue following Western blot analysis. From this, it can be suggested that Sunitinib has the potential to inhibit ATP and result in the inactivation of AMPK phosphorylation, a possible anticancer approach by Sunitinib in Figures 21a and 21b. As mentioned previously, ATP is required for the regulation of cell growth, proliferation and activation of the AMPK signalling pathway; activated during cell starvation of energy to inhibit the biosynthetic pathways of lipid, protein and glycogen synthesis in mammals (Stapleton *et al*. 1996, Webster *et al*. 1999, Doenst *et al*. 2013, Gupta *et al*. 2009, Weiss *et al*. 2005, Ingwall 1993, Bolling *et al*. 1991). To support this, as speculated by Force *et al*. (2007) and as shown by Laderoute *et al*. (2010), Sunitinib results in the inhibition of ATP and thusly contributing towards the prevention of AMPK activation and the release of AMPK-associated downstream targets.

However, the deregulation of mTOR is hypothesised to lead to cell growth and proliferation (Neshat *et al*. 2001), the signalling pathways associated with this are considered to be dysregulated in certain cancers, highlighting the AMPK signalling pathway as an area of interest in anti-cancer. For this, we incubated HepG2 and HL60 cell lines with Metformin at concentrations up to 1 mM in Figures 22a and 22b. The inhibition of the energy salvation pathway via AMPK activation and mTOR inhibition can potentially prevent and decrease cancer growth, an example of this is in breast cancer where the loss of expression of the tumour suppressor PTEN has been demonstrated to lead to uncontrolled activity of mTOR (Neshat *et al*. 2001, Petroulakis *et al*. 2006). An exposure to a growth inhibitory concentration of a drug, such as Metformin in order to indirectly activate AMPK signalling and result in mTOR inhibition can lead to decreased protein synthesis, blocking growth and proliferation of cancer cells (Zakilkhani *et al*. 2006, Dowling *et al*. 2007).

In Section 4.1.-4.7., we demonstrated that Metformin was able to initiate a cardioprotective effect via the activation of the AMPK signalling pathway, as evidenced by the inhibition of adenosine with NBTI. In cancer cell studies, the addition of adenosine was shown to induce apoptosis in human epithelial cancer cells taken from the breast, the colon and the ovary via the intrinsic pathway (Barry

and Lind 2000, Schrier e*t al*. 2001, Sai e*t al.* 2006). Adenosine was taken up into cells via adenosine transporters, highlighting conversion to AMP by adenosine kinase (Barry and Lind 2000, Schrier e*t al*. 2001, Sai e*t al.* 2006). With this, it can be suggested that Metformin may be able to result in an anti–cancer effect via the use of adenosine particularly via the activation of AMPK. However, we demonstrated that up to a concentration of 1 mM, in Figures 22a and 22b, Metformin did not demonstrate any significant reductions in cell viability. In contrast to our approach, the study by Sai *et al*. (2006) demonstrated that extracellular adenosine activation of AMPK (5 mM) was able to increase apoptosis in rat astrocytoma cells and potentially induce cancer cell death, a concentration 5 times greater than the highest dose of Metformin used in our study, and 100 times greater than the concentration of 50 µM used in our animal studies to demonstrate cardioprotection, and therefore would be greater than clinically relevant doses of Metformin.

As previously described, the primary use of Metformin is in the treatment of T2D mellitus, studies have highlighted the potential for Metformin reduce the risk of cancer via the involvement of the AMPK signalling pathway (Evans *et al*. 2005, Zakikhani *et al*. 2006, Ben Sahra *et al*.2008. Results from Figures 22a and 22b demonstrated that Metformin did not result in a significant decrease in live cell population or a reduction in cell viability, it was shown that in our MTT experiment Metformin at the highest concentration 1 mM did not induce apoptosis in HepG2 or HL60 cell lines. To support our results, the study by Ben Sahra *et al*. (2006) demonstrated that Metformin does not induce apoptosis but inhibit the cell cycle at the G_0/G_1 transition phase in DU145, PC–3, LNCaP and P69 cells at concentrations 1 and 5 mM The study by Ben Sahra *et al*. (2008) demonstrated that Metformin had no effect on caspase-3 activity, whilst Metformin at 1 and 5 mM did not affect annexin V positive cells following annexin V-FITC labelling assay in DU145 and PC–3 cells. Following this, it can be suggested that in order to demonstrate Metformin-induced cell death in specific cancer cell lines a concentration greater than 1mM is required. Within the extrinsic pathway A_2 adenosine receptors that are involved with G_2 proteins are said to play a role in apoptosis in cancer cells including myeloid leukaemia cells (Sai e*t al.* 2006). In contrast to A_2 adenosine receptors being linked to an increase in apoptosis, little evidence exists suggesting A_1 adenosine receptors are linked to apoptotic pathways, moreover A_1 adenosine receptors were shown to

attenuate apoptotic cell death (Lee *et al*. 2004, Pingle *et al*. 2004, Regan e*t al*. 2003). The same study by Sai e*t al*. (2006) demonstrated that activation of AMPK was able to reduce cell viability but did not enhance adenosine–induced cytotoxicity, the authors suggested that adenosine was able to induce cell death by a similar mechanism to that of AMPK-induced cell death. The activation of AMPK and agonism of adenosine receptors was shown to activate caspases-3 and -9, suggesting that adenosine is involved in activation of caspases-3 and -9 via A_1 adenosine receptors and the activation of AMPK (Sai *et al*. 2006).

Metformin was shown to inhibit cyclin D1 expression and pRb phosphorylation independent of AMPK (Ben Sahra *et al*. 2008). Cancer cell proliferation and cell growth is controlled by cyclins and cyclin inhibitors, in particular one main checkpoint is the G_1/S transition (Ben Sahra *et al*. 2008). Cyclin D1 is said to play an important role in proliferation by regulating cell cycle machinery, adjusting expression levels to that of the proliferative environment of the cell (Ben Sahra *et al*. 2008). Results from Figures 23a and 23b demonstrated that there was an overall statistical significance between Sunitinib and the co-treatment of Sunitinib and Metformin in both the HepG2 and HL60 cell lines. It was demonstrated in the HepG2 cell line that the combination of Sunitinib with 50 µM Metformin (co–treatment) resulted in an increase in the EC_{50} concentration when compared to the Sunitinib treatment group alone (EC_{50} 34.7 µM for co–treatment vs. 15.4 µM for Sunitinib). Moreover, it was also demonstrated in the HL60 cell line that the combination of Sunitinib with 50 µM (co-treatment) resulted in an increase in EC_{50} concentration when compared to the Sunitinib treatment group alone (EC_{50} 18.2 µM for co–treatment vs. 10 µM for Sunitinib).

Moreover, in the HepG2 cell line, statistical significance was only demonstrated for the co–treatment of Metformin 50 µM and Sunitinib at 30 µM concentration against the Sunitinib at 30 µM concentration. This demonstrates that Metformin is potentially reducing Sunitinib's anti–proliferative ability at this concentration but is not demonstrating a significant change at Sunitinib concentrations lower or greater than 30 µM. From this, it can be suggested that in the HepG2 cell line the addition of Metformin at 50 µM is not having a significant effect on Sunitinib's anti-proliferative properties at the relevant concentration of 1 µM Sunitinib, but at concentrations greater than 1 µM Sunitinib, hence the increase in EC_{50} concertation.

From Figure 25a, the addition of NBTI at 1 µM in combination with Sunitinib and 50 µM Metformin demonstrated a statistical significant increase in cell viability, when compared to the co–treatment group of Metformin 50 µM and Sunitinib at 30 µM concentration, but not during other concentrations of Sunitinib. This suggests that inhibition of AMPK signalling, via the administration of NBTI, is not resulting in an increase in anti-proliferative properties of the co–treatment of Metformin at 50 µM with Sunitinib in the HepG2 cell line. However, as shown by Figure 25a a statistically significant increase in EC_{50} concentration was shown for the co–treatment and NBTI group (EC_{50} 43.3 µM) when compared to the co–treatment group alone (EC_{50} 34.7 µM). To support this, the study by Ben Sahra *et al*. (2008) demonstrated that Metformin has the ability to activate AMPK in human prostate cancer cells but can mediate its effects independently of AMPK. Inhibition of AMPK was shown to not reverse the anti–proliferative properties of Metformin but rather the effects were mediated through the mTOR pathway independently of AMPK, suggesting an alternative approach (Ben Sahra *et al*. 2008). To investigate this we would need to investigate the role of mTOR signalling during the co-treatment of Sunitinib and Metformin, and during the co-treatment of Sunitinib and Metformin and NBTI.

Similarly to the HepG2 cell line, the HL60 cell line from Figure 23b demonstrated that the addition of Metformin at 50 µM (co–treatment) was able to result in an increase in the EC_{50} concentration (EC_{50} 18.2 µM for co–treatment) when compared to Sunitinib treatment alone (EC_{50} 10 µM, $p<0.05$). Results from Figure 23b suggest that the addition of Metformin at 50 µM is resulting in a pro-cell proliferative effect during co–administration with Sunitinib. Moreover, Metformin at 50 µM in co–treatment with Sunitinib at concentrations 10, 30, 60 and 100 µM demonstrated a statistical significant increase in cell viability when compared to Sunitinib treatment alone at concentration 10, 30, 60 and 100 µM ($p<0.05$). However, the addition of Metformin with Sunitinib at the concentration of 1 µM was not statistically significant when compared to Sunitinib at concentration of 1 µM alone ($p>0.05$). This suggests that Metformin is not resulting in an increase in cell viability during co–administration with Sunitinib at 1 µM but at Sunitinib concentrations greater than 1 µM. The addition of NBTI with Sunitinib and Metformin (co–treatment and NBTI) in Figure 25b demonstrated a statistically significant increase in EC_{50} concentration (33 µM) for

co–treatment and NBTI) when compared to the co–treatment group alone (18.3 µM, $p<0.05$).

Many studies have demonstrated Metformin to have an anti-apoptotic effect, and as we have shown, Metformin can be used in co–administration with Sunitinib to reduce Sunitinib–induced cardiac myocyte damage in Section 4. From our previous chapter, it can be suggested that Metformin may be resulting in a similar anti–apoptotic effect during Sunitinib co–treatment in the HL60 and HepG2 cell lines, potentially preventing Sunitinib to initiate an anti–cell proliferative effect in the HL60 and HepG2 cell lines. However, the addition of NBTI at 1 µM with the combination of Sunitinib and Metformin at 50 µM resulted in an increase in the EC_{50} value and an increase in cell proliferation in both the HepG2 and HL60 cell line, when compared to the combination of Sunitinib and Metformin at 50 µM alone. This suggests that the activation of AMPK is having an involvement in preventing Sunitinib's anti-proliferative properties. Moreover, the prevention of Sunitinib's anti-proliferative properties is having a greater effect in the HL60 cell line than in the HepG2 cell line. Moreover, results from Figures 23a-23b and 25a–25b suggest that although AMPK activation is occurring by the addition of Metformin, this is not resulting in a complete inhibition of Sunitinib's anti-proliferative properties, as evidenced by the continued decrease in cell viability during co–administration with 50 µM Metformin and the co–treatment with 50 µM Metformin and NBTI. The inhibition of AMPK signalling was previously suggested to be an indirect mode of Sunitinib's anti-proliferative properties (Force *et al*. 2007). To support this, we previously highlighted that although Sunitinib was able to decrease phosphorylation of AMPK compared to non–treatment, Sunitinib did not result in a complete inhibition of AMPK signalling in Section 4. From this, previous reports and literature have highlighted that the inhibition of AMPK signalling is associated with the release of mTOR signalling and downstream–associated targets, (Laderoute *et* al. 2010, Force *et al*. 2007).

mTOR, alongside the upstream PI3K/Akt signalling pathway, has been associated with proliferation and growth of certain cancer cells particularly leukaemia (HL60) cells (Liu *et* al. 2016, Xu *et al*. 2003, Pathania *et al*. 2013, Sun *et al*. 2011). The mTOR signalling pathway was reported to be upregulated in 40–50 % in hepatocellular samples, whilst inhibition of mTOR was reported to demonstrate

antitumoural activity (Liu *et* al. 2016, Sieghart *et al*. 2007, Sahin *et al*. 2004, Vilanueva *et al*. 2008, Gabrinksi *et al*. 2012). Inhibition of mTOR, and the associated down–stream targets, was demonstrated to result in a pro–apoptotic response of HL60 cells (Pathania *et al*. 2013). The over-activation of the PI3K/Akt/mTOR pathway is associated with the activation of HIF1α, required for the regulation of tumour genesis and angiogenesis (Semenza 2003, Hu *et al.* 2012, Soni and Padwad 2017, Sun *et al*. 2011, Liu *et* al. 2016, Zakikhani *et al*. 2006). It would be hypothesised that the inhibition of AMPK signalling, in Figures 25a and 25b, could have contributed towards the potential activation of mTOR signalling and thereby resulting in an increase in cancer cell proliferation in the HL60 and HepG2 cell lines, but not during incubation with the highest concentration of Sunitinib, however in order to demonstrate this an inhibitor of mTOR signalling such as Temsirolimus would need to be utilised in our study with the combination of Sunitinib and Metformin.

It must be noted that Metformin is a biguanide and does not activate AMPK directly in cell free assays, unlike the compound AICAR, but rather indirectly via the inhibition of the complex -1 of the respiratory chain complex in order to increase the AMP: ATP ratio, the effect of Metformin could be from the result of ATP depletion rather than AMPK activation (Hardie 2003). As discussed, results from the MTT assay would suggest that Sunitinib is resulting in an ATP–decreasing effect in cell lines; the resulting effect would be counter-balanced by Metformin's ability to restore energy deprivation levels via increasing AMP (Foretz and Viollet 2011, Winder and Hardie 1999). Nevertheless, it was demonstrated in the HepG2 and HL60 cell lines that co-administration of Sunitinib with Metformin resulted in an increase in the EC_{50} concentration, however the co-administration did not result in an inhibition of Sunitinib's anti-proliferative properties. The effects of Metformin could be potentially independent of AMPK in the cell lines (Ben Sahra *et al*. 2008), however further work would need carried out to determine the direct role of AMPK and mTOR down–stream targets in co-administration with Sunitinib.

5.5. Conclusion

To conclude, results obtained in Section 5 have demonstrated the use of the MTT assay to investigate the cell proliferative ability of Sunitinib during co–administration with Metformin at 50 µM in HepG2 and HL60 cancer cell lines. Moreover, the combination of Sunitinib with Metformin was administered with the AMPK inhibitor NBTI to investigate the potential for the inhibition of AMPK during activation by Metformin. Results in Section 5.3.1 have demonstrated the increase in EC_{50} concentration for the co–administration of Metformin with Sunitinib when compared to Sunitinib treatment alone, as well as a right-shift of the curve. However, the addition of Metformin did not result in a complete inhibition of Sunitinib's anti–proliferative ability; whilst the addition of Metformin had no statistically significantly effect on Sunitinib at 1 µM concentration, the concentration used in the Langendorff study in Sections 3-4. Metformin and NBTI alone did not demonstrate a significant decrease of cell viability in either HepG2 or HL60 cell lines. The introduction of NBTI demonstrated a statistically significance increase in EC_{50} concentration when compared to the co–treatment groups alone, however this may be resulting from several down-stream targets following AMPK inhibition, such as the proposed activation of mTOR signalling. However, despite causing an increase in EC_{50} concentration, the use of NBTI was only shown to significantly increase cell viability for the co–treatment of Metformin and Sunitinib at 30 µM concentration in the HepG2 cell line, and significantly increased cell viability for the co–treatment of Metformin and Sunitinib at 60 µM concentration in the HL60 cell line. Results obtained could suggest an AMPK–independent effect of Metformin co-treatment, however further investigation is required in order to substantiate this hypothesis.

Chapter Six: General Discussion.

6.1. Summary of findings

The aim of this study was to investigate the potential for Metformin to demonstrate cardioprotective properties when administered together with Sunitinib treatment whilst also investigating the associated intracellular mechanism involved in cardioprotection using *ex-vivo* modelling of the Langendorff system and primary isolated cardiac myocytes. Moreover, we further investigated the associated effects of both Metformin and Sunitinib administration in *in-vitro* cancer cell models involving HepG2 and HL60 cancer cell lines.

In summary, our results demonstrated the effects of cardiotoxicity associated with Sunitinib administration using Langendorff perfused rat hearts and isolated cardiac myocytes. Sunitinib resulted in a significant decrease in haemodynamic function as evidenced by a significant decrease in LVDP in comparison to vehicle control. Moreover, Sunitinib resulted in a significant increase in infarct percentage when compared to vehicle control. The administration of Metformin together with Sunitinib was demonstrated to attenuate this increase in infarct percentage that was caused by Sunitinib administration alone. Moreover, the co-administration of Metformin was shown to restore changes in haemodynamic parameters of LVDP, similar to that of the vehicle control. However, it was further shown that the co-treatment of Metformin and Sunitinib demonstrated a significant decrease in CF at the 160 and 175 minute time points, demonstrating possible vasoconstrictive properties as discussed previously.

Following this, we investigated the intracellular signalling and role of AMPK in Metformin-induced cardioprotection. The use of NBTI in combination with Metformin and Sunitinib inhibited the cardioprotective properties of Metformin, as evidenced by an increase in infarct percentage and a decrease of live cell population of isolated cardiac myocytes. The role of AMPK signalling was demonstrated using Western blot analysis of left atrium tissue following Langendorff perfusion. This was evidenced by an increase in phosphorylated AMPK signalling during co-administration of Metformin with Sunitinib, compared to Sunitinib administration

alone. The increase in phosphorylated AMPK signalling was shown to be attenuated during the combination of Metformin, Sunitinib and NBTI.

Furthermore, it was demonstrated that the combination of Metformin with Sunitinib resulted in an increase in the EC_{50} concentration when compared to Sunitinib treatment alone in both HepG2 and HL60 cancer cell lines. However, this adds to the risk-benefit balance debate, in regards to this the potential benefits of Metformin administration would need to be weighed up against the potential for Metformin to reduce the cytotoxic properties of Sunitinib treatment.

6.2. Considerations and concluding remarks

6.2.1. Mechanism of Sunitinib-induced cardiotoxicity

The presented study warrants further research in regards to the direct mechanism of Sunitinib-induced cardiotoxicity. In particular the direct role of Sunitinib on AMPK signalling would need to be investigated further. It is worth considering if Sunitinib is resulting in a direct inhibition of AMPK signalling as suggested by a majority of literature, or if Sunitinib is resulting in an indirect reduction in AMPK signalling due to ATP-inhibiting properties. Moreover, this would also apply to Sunitinib-induced cardiotoxicity, this could suggest the inhibition of ATP via Sunitinib is resulting in a decrease in cardiac myocyte viability due to a reduction in ATP available, but not resulting in a direct activation of apoptosis or necrosis. For this, it is worth investigating Sunitinib's properties involving the Akt, PI3K and ERK pathways.

6.2.2. Considerations for Metformin use

When administering Metformin, it is worth considering the possibility of nephrotoxicity and lactic acidosis during ST-segment elevation myocardial infarction and reperfusion via primary percutaneous coronary intervention, as mentioned this can arise following prolonged AMPK activation (Bromage and Yellon 2015). In this regard, a risk-benefit balance will be required as well as precautionary safety profile when considering a level 2 study. Furthermore, it was noted by Hardie (2006) in his correspondence letter that the inhibition of the respiratory chain by biguanides accounts for the case of lactic acidosis and that this led to the withdrawal of Phenformin, with the side effect being more common with Phenformin than Metformin. The side effect may be more prominent due to the drug being able to enter cell more rapidly and can build up concentrations sufficient enough to result in a complete inhibition of the respiratory chain, with changes in cellular AMP: ATP more readily observed during incubation of cells with Phenformin but more difficult with Metformin (Hawley *et al*. 2005, Hawley *et al*. 2002, Hardie 2006). It must be reiterated that results observed in this study did not demonstrate toxicity properties during Metformin administration; however a significant decrease in CF was shown during Metformin coadministration with Sunitinib at the 160 and 175 minute time points compared to the vehicle control, highlighting that the use of Metformin as an adjunctive treatment is not without caution as suggested by previous studies discussed in section 3.4. These considerations of Metformin use would need to be considered for further experimentation such as the use of *in-vivo* modelling.

6.2.3. Concluding remarks

As demonstrated, Sunitinib-induced cardiotoxicity was shown to be attenuated using the co-administration with Metformin. However further research is required in regards to the chronic administration of Sunitinib but also if Metformin is suitable for use in *in-vivo* experimentation. Although a good starting point, it is known that Langendorff studies involving coronary organs are not the same as *in-vivo* experimentation. For this, careful considerations need to account for factors such as the health of the animals, age, weight, hormonal and the effects of the administration of Metformin and Sunitinib on other organs. From this, the whole bodily processes such as intracellular stresses could contribute towards toxicity of Sunitinib, whilst liver health and activity in the liver could prevent the cardioprotective properties of Metformin following administration.

When interpreting our data, it is important to understand that when administering Sunitinib and Metformin, a significant number of down-stream effects could be promoted and could contribute towards cell survival and overall survival for patients, however our research focussed primarily on the investigation of AMPK signalling in the heart and the co-administrative effects Metformin and Sunitinib in cancer cell lines. With this in mind, many studies exist regarding the investigation of several pathways, aside from AMPK signalling, with the respective effects on the heart. As touched in Sections 3.1.-5.5., it is always worth investigating the mechanisms of upstream and downstream signalling pathways and associated proteins in order to further evaluate the effects of Metformin when co-administered with Sunitinib. Nevertheless, it must be reiterated that our results demonstrated that Metformin was able to significantly reduce effects of Sunitinib-induced cardiotoxicity in SD rat hearts, associated with AMPK signalling. Although the co-administration was shown to result in an increase in the EC_{50} concentration of Sunitinib when compared to Sunitinib administration alone in cancer cell lines, Metformin did not result in a complete inhibition or prevention of Sunitinib's anti-cancer properties. As mentioned in Section 6.1., this adds to the risk-benefit balance debate and the potential for Metformin to reduce the cytotoxic properties of Sunitinib treatment would need to be discussed in a wider context.

Chapter Seven: Appendix

7.1. The effects of Sunitinib ± Metformin ± NBTI on haemodynamics in 7-8 months female SD rat hearts.

Figure i the effects of Sunitinib in the absence and presence of Metformin and NBTI on HR in 7-8 months female SD rat hearts.

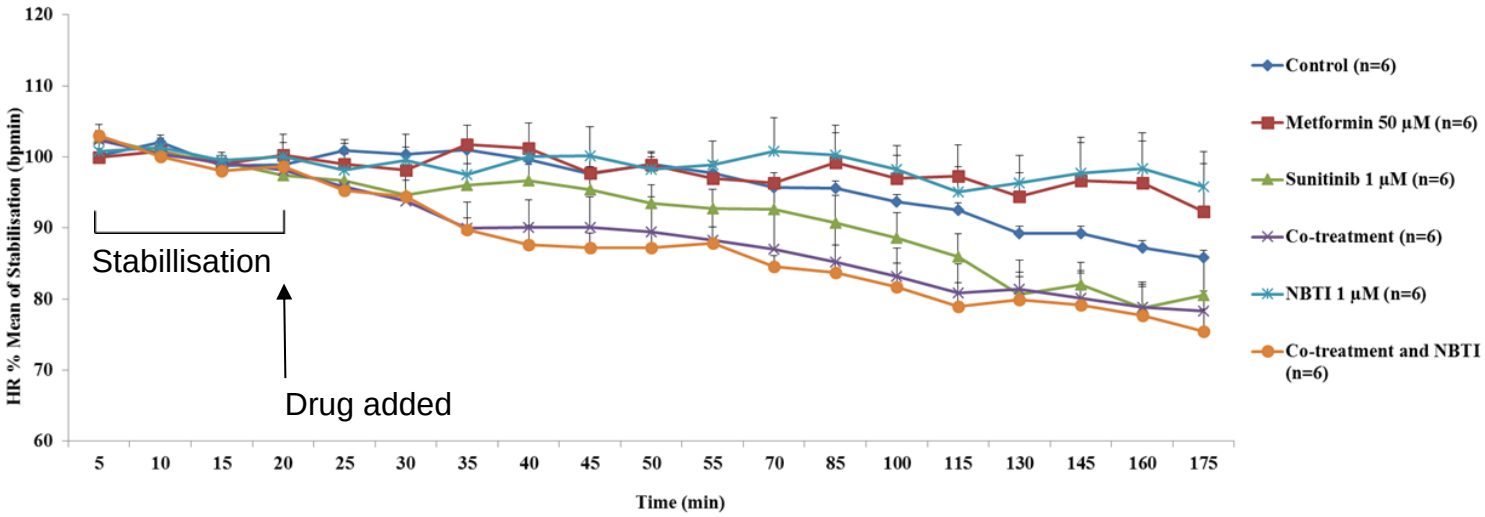

Figure i: The effects of drug treatment on HR as a percentage of mean stabilisation: vehicle control (blue), Metformin (50 µM) (green), Sunitinib (1 µM) (red), the combination of Metformin (50 µM) and Sunitinib (1 µM) (co-treatment, purple), NBTI (1 µM) (turquoise), and the combination of Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM) (co-treatment and NBTI, orange). Data is presented as mean ± SEM of 6 experiments (n=6).

From figure i, it can be seen that there was no overall change in HR at set time points between all treatment groups.

From figure i, single factor ANOVA and post-hoc Tukey and LSD demonstrated no statistical significant changes to HR during the treatment period for the Sunitinib group compared to vehicle control at selected time points ($p>0.05$). Moreover, the combination of Metformin and Sunitinib (cotreatment) group demonstrated no statistical significant changes to HR when compared to Sunitinib treatment at selected time points ($P>0.05$). Furthermore, the combination of Metformin, Sunitinib and NBTI (co-treatment and NBTI) group demonstrated no statistical significant

change to HR when compared to the combination of Metformin and Sunitinib (co-treatment) group at selected time points (P>0.05).

Figure ii The effects of Sunitinib in the absence and presence of Metformin and NBTI on LVDP in 7-8 months female SD rat hearts.

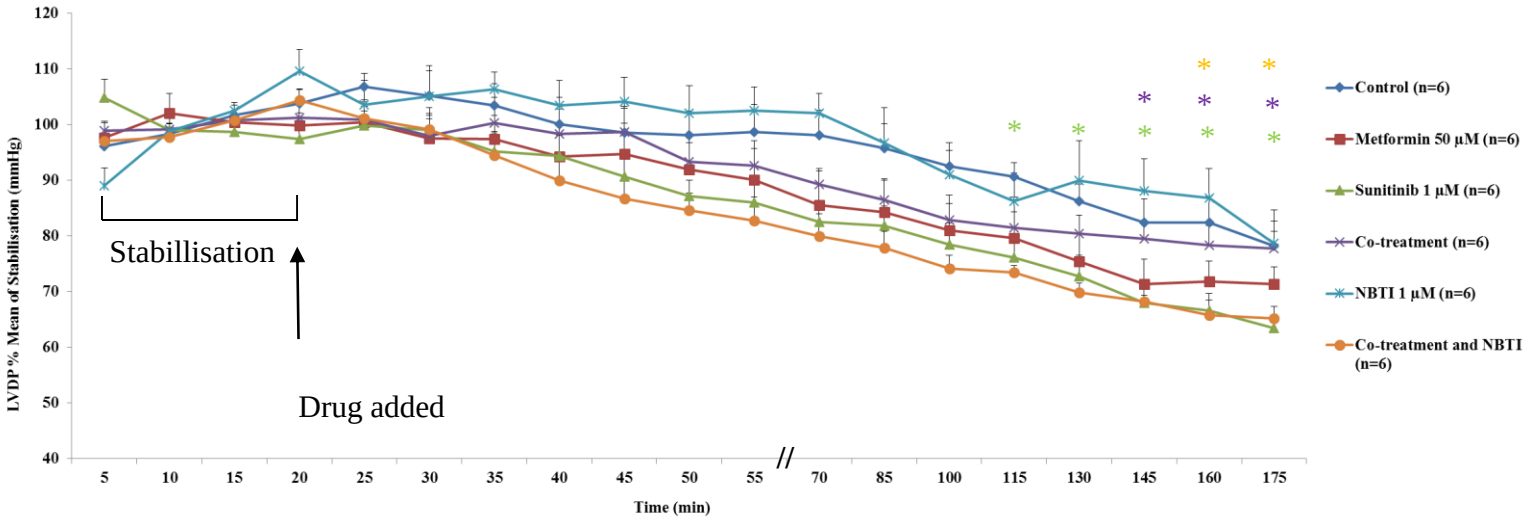

Figure ii: The effects of drug treatment on LVDP as a percentage of mean stabilisation: vehicle control (blue), Metformin (50 µM) (green), Sunitinib (1 µM) (red), the combination of Metformin (50 µM) and Sunitinib (1 µM) (co-treatment, purple), NBTI (1 µM) (turquoise), and the combination of Metformin (50 µM), Sunitinib (1 µM) and NBTI (1 µM) (co-treatment and NBTI, orange). Data is presented as mean ± SEM of 6 experiments (n=6). *Key statistical significance: *=p<0.05 Sunitinib vs vehicle control. *=p<0.05 Sunitinib vs Co-treatment. *=p<0.05 Co-treatment vs Co-treatment and NBTI*.

From Figure ii, it can be seen that there is a decrease in LVDP for the Sunitinib treatment group compared to the vehicle control group at specific time points. Moreover, it can be seen that the combination of Metformin and Sunitinib (co-treatment) group attenuated the Sunitinib-induced decrease in LVDP at specific time points 145, 160 and 175 minutes. Furthermore, the addition of NBTI with Metformin and Sunitinib (co-treatment and NBTI) decreased LVDP when compared to the Metformin and Sunitinib (co-treatment) group at the 160 and 175 minute time points.

From Figure ii, single factor ANOVA with post-hoc Tukey and LSD demonstrated statistical significance for the Sunitinib treatment group against vehicle control at selected time points 115, 130, 145, 160 and 175 minutes ($p<0.05$, 115 minute; 91

± 3 % vs. 76 ± 4 %, 130 minute; 86 ± 3 % vs. 73 ± 4 %, 145 minute; 82 ± 4% vs. 68 ± 3 %, 160 minute; 82 ± 5 % vs. 67 ± 3 %, 175 minute; 64 ± 2 % vs. 78 ± 4 %). Moreover, single factor ANOVA and post-hoc Tukey and LSD demonstrated statistical significance for the Sunitinib and Metformin (co-treatment) group against the Sunitinib treatment group at selected time points 145, 160 and 175 minutes ($p<0.05$, 145 minute; 80 ± 3 % vs. 68 ± 3 %, 160 minute; 78 ± 4 % vs. 67 ± 3 %, 175 minute; 78 ± 3 % vs. 64 ± 2 %). Furthermore, single factor ANOVA with post-hoc Tukey and LSD demonstrated statistical significance for the Sunitinib, Metformin and NBTI (co-treatment and NBTI) treatment group against the Sunitinib and Metformin (co-treatment) group at selected time points 160 and 175 minutes ($p<0.05$, 160 minute; 66 ± 3% vs. 78 ± 4 %, 175 minute; 65 ± 2 % vs. 78 ± 3 %).

Figure iii The effects of Sunitinib in the absence and presence of Metformin and NBTI on CF in 7-8 months female SD rat hearts.

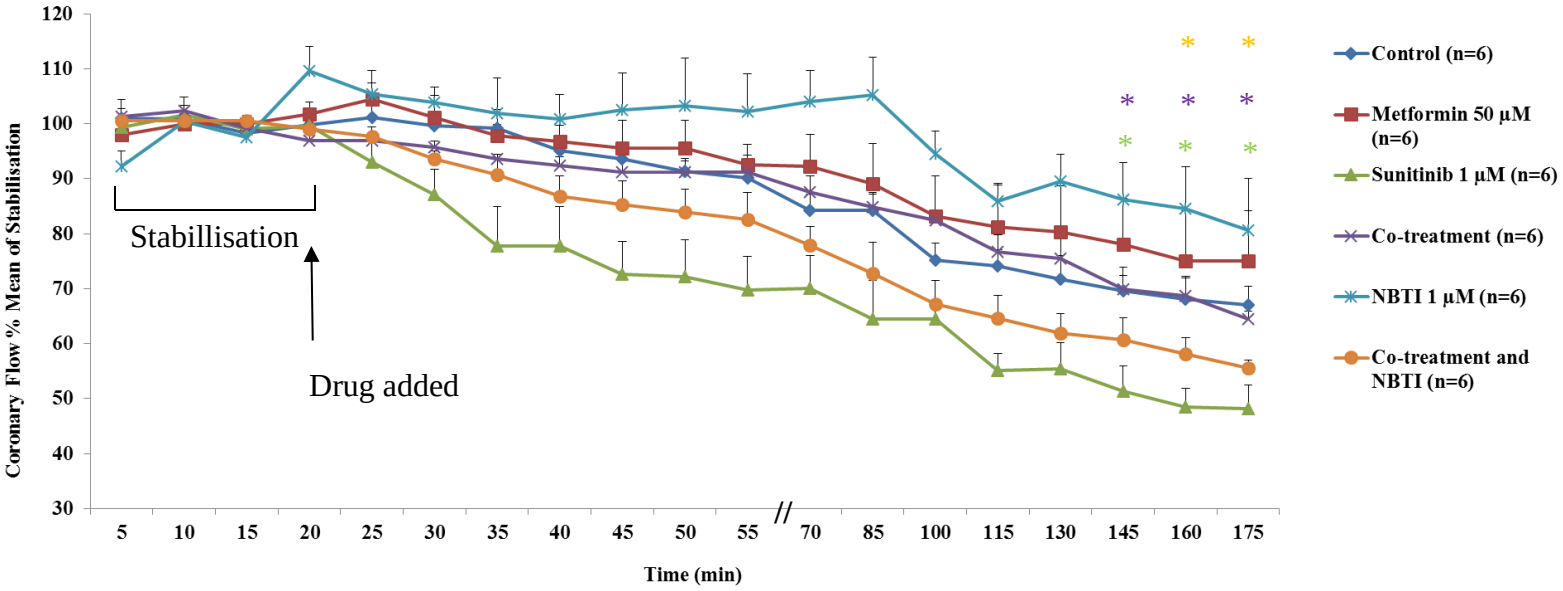

Figure iii: The effects of drug treatment on CF as a percentage of mean stabilisation: vehicle control (blue), Metformin (50µM) (green), Sunitinib (1µM) (red), the combination of Metformin (50µM) and Sunitinib (1µM) (co-treatment, purple), NBTI (1µM) (turquoise), and the combination of Metformin (50µM), Sunitinib (1µM) and NBTI (1µM) (co-treatment and NBTI, orange). Data is presented as mean ± SEM of 6 experiments (n=6). *Key statistical significance: *=p<0.05 Sunitinib vs vehicle control. *=p<0.05 Sunitinib vs Co-treatment. *=p<0.05 Co-treatment vs Co-treatment and NBTI*.

From figure iii, it can be seen that the Sunitinib treatment group decreased CF when compared to the vehicle control group at certain time points. Moreover, the Sunitinib-induced decrease in CF was shown to be attenuated at specific time points following the combination of Metformin and Sunitinib (co-treatment), when compared to the Sunitinib treatment group. Furthermore, the addition of NBTI with Metformin and Sunitinib (co-treatment and NBTI) was shown to decrease CF when compared to the Metformin and Sunitinib (co-treatment) group at specific time points.

From figure iii, single factor ANOVA with post-hoc Tukey and LSD demonstrated statistical significance for the Sunitinib treatment group against vehicle control at

selected time points 145, 160 and 175 minutes ($p<0.05$, 145 minute; 51 ± 5 % vs. 70 ± 4 %, 160 minute; 48 ± 3 % vs. 68 ± 4 %, 175 minute; 48 ± 4 % vs. 67 ± 4 %). Moreover single factor ANOVA and post-hoc Tukey and LSD demonstrated statistical significance for the Sunitinib and Metformin (co-treatment) group against the Sunitinib treatment group at selected time points 145, 160 and 175 minutes ($p<0.05$, 145 minute; 70 ± 2 % vs. 51 ± 5 %, 160 minute; 69 ± 3 % vs. 48 ± 3 %, 175 minute; 64 ± 1 % vs. 48 ± 4 %). Furthermore, single factor ANOVA with post-hoc Tukey and LSD demonstrated statistical significance for the Sunitinib, Metformin and NBTI (co-treatment and NBTI) treatment group against the Sunitinib and Metformin (co-treatment) group at selected time points 160 and 175 minutes ($p<0.05$, 160 minute; 58 ± 3 % vs. 69 ± 3 %, 175 minute; 56 ± 1 % vs. 64 ± 1 %).

7.2. The effects of Sunitinib ± Metformin ± NBTI on infarct percentage in 7-8 months female SD rat hearts.

Figure iv the effects of Sunitinib in the absence and presence of Metformin and NBTI on infarct percentage in 7-8 months female SD rat hearts.

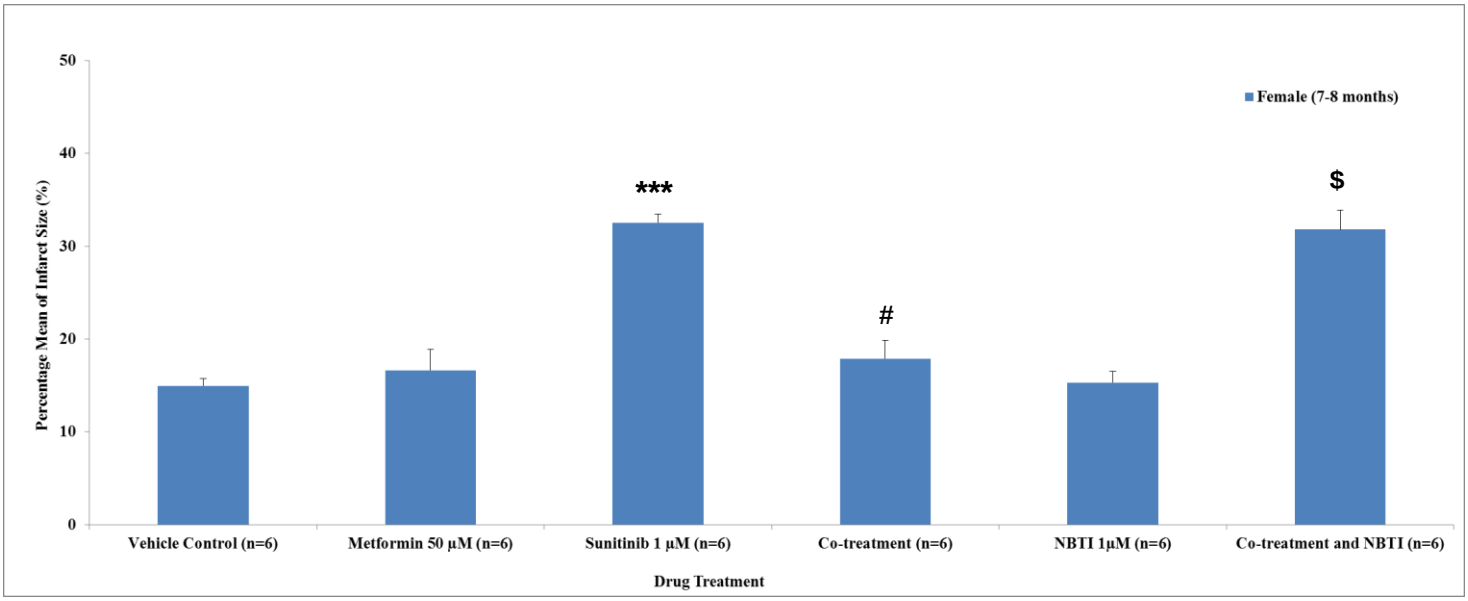

Figure iv: The effects of vehicle control, Metformin (50µM), Sunitinib (1µM), the combination of Metformin and Sunitinib (Co-treatment), NBTI (1µM) and the combination of Metformin, Sunitinib and NBTI on infarct percentage (%). Data is presented as mean ±SEM of 6 experiments (n=6). *Key statistical significance: ***=p<0.001 Sunitinib vs vehicle control. #=p<0.05 Co-treatment vs Sunitinib, $=p<0.05 Co-treatment and NBTI vs Co-treatment.*

From figure iv, it can be seen that the Sunitinib treatment group resulted in an increase in infarct percentage when compared to the vehicle control. Moreover, the co-treatment group was shown to attenuate the Sunitinib-induced increase in infarct percentage. Furthermore, the addition of NBTI with Metformin and Sunitinib (co-treatment and NBTI) was shown to attenuate the decrease in infarct percentage when compared to the co-treatment group.

From figure iv, single factor ANOVA with post-hoc Tukey and LSD demonstrated statistical significance between the Sunitinib treatment group compared to the vehicle control ($p<0.05$, 33 ± 1 % vs. 15 ± 1 %). Moreover, statistical significance

was shown for the Metformin and Sunitinib (co-treatment) treatment group when compared to the Sunitinib treatment group ($p<0.05$, 18 ± 2 % vs. 33 ± 1 %). Furthermore, statistical significance was shown for the Metformin, Sunitinib and NBTI (co-treatment and NBTI) group when compared to the Metformin and Sunitinib (co-treatment) group ($p<0.05$, 18 ± 2 % vs. 32 ± 2 %).

References

Abdel-Aziz, A. K., Mantawy, E. M., Said, R. S., and Helwa, R. (2016) 'The Tyrosine Kinase Inhibitor, Sunitinib Malate, Induces Cognitive Impairment in-Vivo Via Dysregulating VEGFR Signalling, Apoptotic and Autophagic Machineries'*. Experimental Neurology* 283, 129-141.

Abrams, T. J., Lee, L. B., Murray, L. J., Pryer, N. K., and Cherrington, J. M. (2003) 'SU11248 Inhibits KIT and Platelet-Derived Growth Factor Receptor Beta in Preclinical Models of Human Small Cell Lung Cancer'*. Molecular Cancer Therapeutics* 2 (5), 471-478.

Aguan, K., Scott, J., See, C. G., and Sarkar, N. H. (1994) 'Characterization and Chromosomal Localization of the Human Homologue of a Rat AMP-Activated Protein Kinase-Encoding Gene: A Major Regulator of Lipid Metabolism in Mammals'*. Gene* 149 (2), 345-350.

Alameddine, R. S., Yakan, A. S., Skouri, H., Mukherji, D., Temraz, S., and Shamseddine, A. (2015) 'Cardiac and Vascular Toxicities of Angiogenesis Inhibitors: The Other Side of the Coin'*. Critical Reviews in oncology/hematology* 96 (2), 195-205.

Aleman, B. M., Moser, E. C., Nuver, J., Suter, T. M., Maraldo, M. V., Specht, L., Vrieling, C., and Darby, S. C. (2014) 'Cardiovascular Disease After Cancer Therapy'*. European Journal of Cancer Supplements* 12 (1), 18-28.

Alexander, A., Cai, S. L., Kim, J., Nanez, A., Sahin, M., MacLean, K. H., Inoki, K., Guan, K. L., Shen, J., Person, M. D., Kusewitt, D., Mills, G. B., Kastan, M. B., and Walker, C. L. (2010) 'ATM Signals to TSC2 in the Cytoplasm to Regulate mTORC1 in Response to ROS'*. Proceedings of the National Academy of Sciences of the United States of America* 107 (9), 4153-4158.

Algire, C., Amrein, L., Bazile, M., David, S., Zakikhani, M., and Pollak, M. (2011) 'Diet and Tumor LKB1 Expression Interact to Determine Sensitivity to Anti-Neoplastic Effects of Metformin in Vivo'*. Oncogene* 30 (10), 1174-1182.

Algire, C., Moiseeva, O., Deschenes-Simard, X., Amrein, L., Petruccelli, L., Birman, E., Viollet, B., Ferbeyre, G., and Pollak, M. N. (2012) 'Metformin Reduces Endogenous Reactive Oxygen Species and Associated DNA Damage'. *Cancer Prevention Research (Philadelphia, Pa.)* 5 (4), 536-543.

Alimova, I. N., Liu, B., Fan, Z., Edgerton, S. M., Dillon, T., Lind, S. E., and Thor, A. D. (2009) 'Metformin Inhibits Breast Cancer Cell Growth, Colony Formation and Induces Cell Cycle Arrest in Vitro'. *Cell Cycle* 8 (6), 909-915.

Allard, M. F., Parsons, H. L., Saeedi, R., Wambolt, R. B., and Brownsey, R. (2007) 'AMPK and Metabolic Adaptation by the Heart to Pressure Overload'. *American Journal of Physiology-Heart and Circulatory Physiology* 292 (1), H140-H148.

Altarejos, J. Y. and Montminy, M. (2011) 'CREB and the CRTC Co-Activators: Sensors for Hormonal and Metabolic Signals'. *Nature Reviews Molecular Cell Biology* 12 (3), 141-151.

American Cancer Society (2015), 'What are the key statistics about liver cancer?' *American Cancer Society* [online] available from <http://www.cancer.org/cancer/livercancer/detailedguide/liver-cancer-what-is-key-statistics> [25th August 2015].

An, D., Kewalramani, G., Chan, J., Qi, D., Ghosh, S., Pulinilkunnil, T., Abrahani, A., Innis, S., and Rodrigues, B. (2006) 'Metformin Influences Cardiomyocyte Cell Death by Pathways that are Dependent and Independent of Caspase-3'. *Diabetologia* 49 (9), 2174-2184.

Anastasiou, D. (2013) 'Metformin: A Case of Divide and Conquer'. *Breast Cancer Research: BCR* 15 (2), 306.

Andrews, M., Soto, N., and Arredondo, M. (2012) 'Effect of Metformin on the Expression of Tumor Necrosis Factor-Alpha, Toll Like Receptors 2/4 and C Reactive Protein in Obese Type-2 Diabetic Patients'. *Revista Medica De Chile* 140 (11), 1377-1382.

Andrzejewski, S., Gravel, S., Pollak, M., and St-Pierre, J. (2014) 'Metformin Directly Acts on Mitochondria to Alter Cellular Bioenergetics'. *Cancer & Metabolism* 2 (1), 12.

Anfossi, G., Russo, I., Bonomo, K., and Trovati, M. (2010) 'The Cardiovascular Effects of Metformin: Further Reasons to Consider an Old Drug as a Cornerstone in the Therapy of Type 2 Diabetes Mellitus'. *Current Vascular Pharmacology* 8 (3), 327-337.

Anjum, R. and Blenis, J. (2008) 'The RSK Family of Kinases: Emerging Roles in Cellular Signalling'. *Nature Reviews Molecular Cell Biology* 9 (10), 747-758.

Aoki, M., Nata, T., Morishita, R., Matsushita, H., Nakagami, H., Yamamoto, K., Yamazaki, K., Nakabayashi, M., Ogihara, T., and Kaneda, Y. (2001) 'Endothelial Apoptosis Induced by Oxidative Stress through Activation of NF-kappaB: Antiapoptotic Effect of Antioxidant Agents on Endothelial Cells'. *Hypertension (Dallas, Tex.: 1979)* 38 (1), 48-55.

Arad, M., Seidman, C. E., and Seidman, J. G. (2007) 'AMP-Activated Protein Kinase in the Heart: Role during Health and Disease'. *Circulation Research* 100 (4), 474-488.

Argaud, L., Gateau-Roesch, O., Chalabreysse, L., Gomez, L., Loufouat, J., Thivolet-Bejui, F., Robert, D., and Ovize, M. (2004) 'Preconditioning Delays Ca2+-Induced Mitochondrial Permeability Transition'. *Cardiovascular Research*61 (1), 115-122.

Arsham, A. M., Howell, J. J., and Simon, M. C. (2003) 'A Novel Hypoxia-Inducible Factor-Independent Hypoxic Response Regulating Mammalian Target of Rapamycin and its Targets'. *The Journal of Biological Chemistry* 278 (32), 29655-29660.

Ashall, V. and Dawes, T. (2008) 'Metformin and Lactic Acidosis'. *British Journal of Anaesthesia* 101 (6), 876-877

Ashkenazi, A. (2008) 'Directing Cancer Cells to Self-Destruct with Pro-Apoptotic Receptor Agonists'. *Nature Reviews Drug Discovery* 7 (12), 1001.

Avogaro, A., de Kreutzenberg, S. V., and Fadini, G. P. (2010) 'Insulin Signaling and Life Span'. *Pflügers Archiv-European Journal of Physiology* 459 (2), 301-314.

Aymerich, I., Foufelle, F., Ferre, P., Casado, F. J., and Pastor-Anglada, M. (2006) 'Extracellular Adenosine Activates AMP-Dependent Protein Kinase (AMPK)'. *Journal of Cell Science* 119 (Pt 8), 1612-1621.

Bäcklund, T., Palojoki, E., Saraste, A., Eriksson, A., Finckenberg, P., Kytö, V., Lakkisto, P., Mervaala, E., Voipio-Pulkki, L., and Laine, M. (2004) 'Sustained Cardiomyocyte Apoptosis and Left Ventricular Remodelling After Myocardial Infarction in Experimental Diabetes'. *Diabetologia* 47 (2), 325-330.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., Klevernic, I., Arthur, J. S., Alessi, D. R., and Cohen, P. (2007) 'The Selectivity of Protein Kinase Inhibitors: A further Update'. *The Biochemical Journal*408 (3), 297-315.

Baines, C. P. and Molkentin, J. D. (2005) 'STRESS Signaling Pathways that Modulate Cardiac Myocyte Apoptosis'. *Journal of Molecular and Cellular Cardiology* 38 (1), 47-62.

Baldwin, S. A., Beal, P. R., Yao, S. Y., King, A. E., Cass, C. E., and Young, J. D. (2004) 'The Equilibrative Nucleoside Transporter Family, SLC29'. *Pflügers Archiv* 447 (5), 735-743.

Balen, A. H., Conway, G. S., Kaltsas, G., Techatrasak, K., Manning, P. J., West, C., and Jacobs, H. S. (1995) 'Polycystic Ovary Syndrome: The Spectrum of the Disorder in 1741 Patients'. *Human Reproduction (Oxford, England)* 10 (8), 2107-2111.

Bagnoli, M., Canevari, S., and Mezzanzanica, D. (2010) 'Cellular FLICE-Inhibitory Protein (c-FLIP) Signalling: A Key Regulator of Receptor-Mediated Apoptosis in Physiologic Context and in Cancer'. *The International Journal of Biochemistry & Cell Biology* 42 (2), 210-213.

Bantscheff, M., Eberhard, D., Abraham, Y., Bastuck, S., Boesche, M., Hobson, S., Mathieson, T., Perrin, J., Raida, M., and Rau, C. (2007) 'Quantitative Chemical

Proteomics Reveals Mechanisms of Action of Clinical ABL Kinase Inhibitors'. *Nature Biotechnology* 25 (9), 1035-1044.

Baradari, A. G., Habibi, M. R., Khezri, H. D., Aarabi, M., Khademloo, M., Jalali, Z., and Ghafari, R. (2011) 'Does High-Dose Metformin Cause Lactic Acidosis in Type 2 Diabetic Patients After CABG Surgery? A Double Blind Randomized Clinical Trial'. *Heart International* 6 (1), hi. 2011. e8

Bar-Peled, L., Schweitzer, L. D., Zoncu, R., and Sabatini, D. M. (2012) 'Ragulator is a GEF for the Rag GTPases that Signal Amino Acid Levels to mTORC1'. *Cell* 150 (6), 1196-1208.

Baron, S. J., Li, J., Russell, R. R.,3rd, Neumann, D., Miller, E. J., Tuerk, R., Wallimann, T., Hurley, R. L., Witters, L. A., and Young, L. H. (2005) 'Dual Mechanisms Regulating AMPK Kinase Action in the Ischemic Heart'. *Circulation Research* 96 (3), 337-345.

Barros, L., Hermosilla, T., and Castro, J. (2001) 'Necrotic Volume Increase and the Early Physiology of Necrosis'. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 130 (3), 401-409.

Barreto-Torres, G., Parodi-Rullán, R., and Javadov, S. (2012) 'The Role of PPARα in Metformin-Induced Attenuation of Mitochondrial Dysfunction in Acute Cardiac Ischemia/Reperfusion in Rats'. *International Journal of Molecular Sciences* 13 (6), 7694-7709.

Bell, R. M., Mocanu, M. M., and Yellon, D. M. (2011) 'Retrograde Heart Perfusion: The Langendorff Technique of Isolated Heart Perfusion'. *Journal of Molecular and Cellular Cardiology* 50 (6), 940-950.

Ben Sahra, I., Laurent, K., Loubat, A., Giorgetti-Peraldi, S., Colosetti, P., Auberger, P., Tanti, J., Le Marchand-Brustel, Y., and Bost, F. (2008) 'The Antidiabetic Drug Metformin Exerts an Antitumoral Effect in Vitro and in Vivo through a Decrease of Cyclin D1 Level'. *Oncogene* 27 (25), 3576.

Bergman, R. N., Prager, R., Volund, A., and Olefsky, J. M. (1987) 'Equivalence of the Insulin Sensitivity Index in Man Derived by the Minimal Model Method and the Euglycemic Glucose Clamp'. *The Journal of Clinical Investigation* 79 (3), 790-800.

Beri, R. K., Marley, A. E., See, C. G., Sopwith, W. F., Aguan, K., Carling, D., Scott, J., and Carey, F. (1994) 'Molecular Cloning, Expression and Chromosomal Localisation of Human AMP-Activated Protein Kinase'. *FEBS Letters* 356 (1), 117-121.

Bertrand, L., Ginion, A., Beauloye, C., Hebert, A. D., Guigas, B., Hue, L., and Vanoverschelde, J. L. (2006) 'AMPK Activation Restores the Stimulation of Glucose Uptake in an in Vitro Model of Insulin-Resistant Cardiomyocytes Via the Activation of Protein Kinase B'. *American Journal of Physiology.Heart and Circulatory Physiology* 291 (1), H239-50.

Bhalla, R. C., Toth, K. F., Tan, E., Bhatty, R. A., Mathias, E., and Sharma, R. V. (1996) 'Vascular Effects of Metformin Possible Mechanisms for its Antihypertensive Action in the Spontaneously Hypertensive Rat'. *American Journal of Hypertension* 9 (6), 570-576.

Bhamra, G. S., Hausenloy, D. J., Davidson, S. M., Carr, R. D., Paiva, M., Wynne, A. M., Mocanu, M. M., and Yellon, D. M. (2008) 'Metformin Protects the Ischemic Heart by the Akt-Mediated Inhibition of Mitochondrial Permeability Transition Pore Opening'. *Basic Research in Cardiology* 103 (3), 274-284.

Bhatia, M. (2004) 'Apoptosis versus Necrosis in Acute Pancreatitis'. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 286 (2), G189-G196.

Bianchi, A., Evans, J. L., Iverson, A. J., Nordlund, A. C., Watts, T. D., and Witters, L. A. (1990) 'Identification of an Isozymic Form of Acetyl-CoA Carboxylase'. *The Journal of Biological Chemistry* 265 (3), 1502-1509.

Bickerton, A. S., Clark, N., Meeking, D., Shaw, K. M., Crook, M., Lumb, P., Turner, C., and Cummings, M. H. (2005) 'Cardiovascular Risk in Women with Polycystic Ovarian Syndrome (PCOS)'. *Journal of Clinical Pathology* 58 (2), 151-154.

Birnie, G. D. (1988) 'The HL60 Cell Line: A Model System for Studying Human Myeloid Cell Differentiation'. *The British Journal of Cancer.Supplement* 9, 41-45.

Bischoff, F., Sahyun, M., and Long, M. L. (1929) 'Guanidine Structure and Hypoglycemia'. *Journal of Biological Chemistry* 81 (2), 325-349.

Blandino, G., Valerio, M., Cioce, M., Mori, F., Casadei, L., Pulito, C., Sacconi, A., Biagioni, F., Cortese, G., and Galanti, S. (2012) 'Metformin Elicits Anticancer Effects through the Sequential Modulation of DICER and c-MYC'. *Nature Communications* 3, 865.

Blatherwick, N., Sahyun, M., and Hill, E. (1927) 'Some Effects of Synthalin on Metabolism'. *Journal of Biological Chemistry* 75 (3), 671-683.

BMJ (2016) 'QT Interval and Drug Therapy' *British Medical Journal Drug and Therapeutics Bulletin 1* [online] available from <https://www.bmj.com/content/353/bmj.i2732> [1st June 2019].

Bolling, S. F., Bove, E. L., and Gallagher, K. P. (1991) 'ATP Precursor Depletion and Postischemic Myocardial Recovery'. *Journal of Surgical Research* 50 (6), 629-633.

Bolster, D. R., Crozier, S. J., Kimball, S. R., and Jefferson, L. S. (2002) 'AMP-Activated Protein Kinase Suppresses Protein Synthesis in Rat Skeletal Muscle through Down-Regulated Mammalian Target of Rapamycin (mTOR) Signaling'. *The Journal of Biological Chemistry* 277 (27), 23977-23980.

Borchmann P., Eichenauer D.A., Engert A. State of the art in the treatment of Hodgkin lymphoma. Nat Rev Clin Oncol. 2012;9(8):450–459.

Boussageon, R., Supper, I., Bejan-Angoulvant, T., Kellou, N., Cucherat, M., Boissel, J., Kassai, B., Moreau, A., Cueyffier, F., and Cornu, C. (2012) 'Reappraisal of Metformin Efficacy in the Treatment of Type 2 Diabetes: A Meta-Analysis of Randomised Controlled Trials'. *PLoS Medicine* 9 (4), 465.

Bovelli, D., Plataniotis, G., Roila, F., and ESMO Guidelines Working Group (2010) 'Cardiotoxicity of Chemotherapeutic Agents and Radiotherapy-Related Heart Disease: ESMO Clinical Practice Guidelines'. *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO* 21 Suppl 5, v277-82.

Bowker, S. L., Majumdar, S. R., Veugelers, P., and Johnson, J. A. (2006) 'Increased Cancer-Related Mortality for Patients with Type 2 Diabetes Who use Sulfonylureas or Insulin'. *Diabetes Care* 29 (2), 254-258.

Bristol-Myers Squibb (2018) 'GLUCOPHAGE® Package Inserts', *Bristol-Myers Squibb* [online] available from < https://packageinserts.bms.com/pi/pi_glucophage.pdf> [1st June 2019].

British Heart Foundation (2018) 'CVD Statistics – BHF UK Factsheet', *British Heart Foundation* [online] available from <https://www.bhf.org.uk/-/media/files/research/heart-statistics/bhf-cvd-statistics---uk-factsheet.pdf> [26th June 2018].

Bromage, D. I. and Yellon, D. M. (2015) 'The Pleiotropic Effects of Metformin: Time for Prospective Studies'. *Cardiovascular Diabetology* 14 (1), 109.

Brown, A. M. and Rampe, D. (2000) 'Drug-Induced Long QT Syndrome: Is HERG the Root of all Evil'. *Pharmaceutical News* 7 (4), 75.

Brugarolas, J., Lei, K., Hurley, R. L., Manning, B. D., Reiling, J. H., Hafen, E., Witters, L. A., Ellisen, L. W., and Kaelin, W. G.,Jr (2004) 'Regulation of mTOR Function in Response to Hypoxia by REDD1 and the TSC1/TSC2 Tumor Suppressor Complex'. *Genes & Development* 18 (23), 2893-2904.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) 'Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor'. *Cell* 96 (6), 857-868.

Brunmair, B., Staniek, K., Gras, F., Scharf, N., Althaym, A., Clara, R., Roden, M., Gnaiger, E., Nohl, H., Waldhausl, W., and Furnsinn, C. (2004) 'Thiazolidinediones, Like Metformin, Inhibit Respiratory Complex I: A Common Mechanism Contributing to their Antidiabetic Actions?'. *Diabetes* 53 (4), 1052-1059.

Buja, L. M. (2005) 'Myocardial Ischemia and Reperfusion Injury'. *Cardiovascular Pathology* 14 (4), 170-175.

Bulhak, A. A., Jung, C., Ostenson, C. G., Lundberg, J. O., Sjoquist, P. O., and Pernow, J. (2009) 'PPAR-Alpha Activation Protects the Type 2 Diabetic Myocardium Against Ischemia-Reperfusion Injury: Involvement of the PI3-Kinase/Akt and NO Pathway'. *American Journal of Physiology.Heart and Circulatory Physiology* 296 (3), H719-27.

Buzzai, M., Jones, R. G., Amaravadi, R. K., Lum, J. J., DeBerardinis, R. J., Zhao, F., Viollet, B., and Thompson, C. B. (2007) 'Systemic Treatment with the Antidiabetic Drug Metformin Selectively Impairs p53-Deficient Tumor Cell Growth'. *Cancer Research* 67 (14), 6745-6752.

Calvert, J. W., Gundewar, S., Jha, S., Greer, J. J., Bestermann, W. H., Tian, R., and Lefer, D. J. (2008) 'Acute Metformin Therapy Confers Cardioprotection against Myocardial Infarction Via AMPK-eNOS-Mediated Signaling'. *Diabetes* 57 (3), 696-705.

Camara, A. K., Lesnefsky, E. J., and Stowe, D. F. (2010) 'Potential Therapeutic Benefits of Strategies Directed to Mitochondria'. *Antioxidants & Redox Signaling* 13 (3), 279-347.

Capano, M. and Crompton, M. (2006) 'Bax Translocates to Mitochondria of Heart Cells during Simulated Ischaemia: Involvement of AMP-Activated and p38 Mitogen-Activated Protein Kinases'. *The Biochemical Journal* 395 (1), 57-64.

Cardinale, D., Colombo, A., Sandri, M. T., Lamantia, G., Colombo, N., Civelli, M., Martinelli, G., Veglia, F., Fiorentini, C., and Cipolla, C. M. (2006) 'Prevention of High-Dose Chemotherapy-Induced Cardiotoxicity in High-Risk Patients by Angiotensin-Converting Enzyme Inhibition'. *Circulation* 114 (23), 2474-2481.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) 'Regulation of Cell Death Protease Caspase-9 by Phosphorylation'. *Science (New York, N.Y.)* 282 (5392), 1318-1321.

Carling, D., Fryer, L. G., Woods, A., Daniel, T., Jarvie, S. L., and Whitrow, H. (2003) 'Bypassing the glucose/fatty Acid Cycle: AMP-Activated Protein Kinase'. *Biochemical Society Transactions* 31 (Pt 6), 1157-1160.

Carling, D. (2005) 'AMP-Activated Protein Kinase: Balancing the Scales'. *Biochimie* 87 (1), 87-91.

Carrera, A. C. (2004) 'TOR Signaling in Mammals'. *Journal of Cell Science* 117 (Pt 20), 4615-4616.

Catino, A. B., Hubbard, R. A., Chirinos, J. A., Townsend, R., Keefe, S., Haas, N. B., Puzanov, I., Fang, J. C., Agarwal, N., and Hyman, D. (2018) 'Longitudinal Assessment of Vascular Function with Sunitinib in Patients with Metastatic Renal Cell Carcinoma'. *Circulation: Heart Failure* 11 (3), e004408.

Centres for Disease Control and Prevention (2016) "Defining Adult Overweight and Obesity" *Centres for Disease Control and Prevention* [online] available from <https://www.cdc.gov/obesity/adult/defining.html> [26th June 2018].

Cerda-Kohler H, Henríquez-Olguín C, Casas M, Jensen TE, Llanos P, Jaimovich E. (2018) 'Lactate administration activates the ERK 1/2, mTORC 1, and AMPK pathways differentially according to skeletal muscle type in mouse'. *Physiological reports* 6(18):e13800.

Cezur M, Celmen N, Cetinbas R, Badalov P, Erdemli O. (2009) 'A clinical case of lactic acidosis development in a diabetic patient taking metformin'. *Anesteziol Reanimatol*, (2)(2):74-76.

Cha, H., Choi, J. H., Kim, Y., Kim, J., Ahn, M., and Park, S. (2010) 'Metformin Inhibits Isoproterenol-Induced Cardiac Hypertrophy in Mice'. *The Korean Journal of Physiology & Pharmacology* 14 (6), 377-384.

Chantawansri, C., Huynh, N., Yamanaka, J., Garfinkel, A., Lamp, S. T., Inoue, M., Bridge, J. H., and Goldhaber, J. I. (2008) 'Effect of Metabolic Inhibition on Couplon Behaviour in Rabbit Ventricular Myocytes'. *Biophysical Journal* 94 (5), 1656-1666.

Chen, M. H., Kerkela, R., and Force, T. (2008) 'Mechanisms of Cardiac Dysfunction Associated with Tyrosine Kinase Inhibitor Cancer Therapeutics'. *Circulation* 118 (1), 84-95.

Chen, G., Xu, S., Renko, K., and Derwahl, M. (2012) 'Metformin Inhibits Growth of Thyroid Carcinoma Cells, Suppresses Self-Renewal of Derived Cancer Stem Cells,

and Potentiates the Effect of Chemotherapeutic Agents'. *The Journal of Clinical Endocrinology & Metabolism* 97 (4), E510-E520.

Chen, G., Nicula, D., Renko, K., and Derwahl, M. (2015) 'Synergistic Anti-Proliferative Effect of Metformin and Sorafenib on Growth of Anaplastic Thyroid Cancer Cells and their Stem Cells'. *Oncology Reports* 33 (4), 1994-2000.

Cheung, P. C., Salt, I. P., Davies, S. P., Hardie, D. G., and Carling, D. (2000) 'Characterization of AMP-Activated Protein Kinase Gamma-Subunit Isoforms and their Role in AMP Binding'. *The Biochemical Journal* 346 Pt 3, 659-669.

Chintalgattu, V., Ai, D., Langley, R. R., Zhang, J., Bankson, J. A., Shih, T. L., Reddy, A. K., Coombes, K. R., Daher, I. N., Pati, S., Patel, S. S., Pocius, J. S., Taffet, G. E., Buja, L. M., Entman, M. L., and Khakoo, A. Y. (2010) 'Cardiomyocyte PDGFR-Beta Signaling is an Essential Component of the Mouse Cardiac Response to Load-Induced Stress'. *The Journal of Clinical Investigation* 120 (2), 472-484.

Chintalgattu, V., Rees, M. L., Culver, J. C., Goel, A., Jiffar, T., Zhang, J., Dunner, K., Pati, S., Bankson, J. A., and Pasqualini, R. (2013) 'Coronary Microvascular Pericytes are the Cellular Target of Sunitinib malate–induced Cardiotoxicity'. *Science Translational Medicine* 5 (187), 187ra69-187ra69.

Chiang, G. G. and Abraham, R. T. (2007) 'Targeting the mTOR Signalling Network in Cancer'. *Trends in Molecular Medicine* 13 (10), 433-442.

Choueiri TK, Schutz FA, Je Y, Rosenberg JE, Bellmunt J. Risk of arterial thromboembolic
events with sunitinib and sorafenib: a systematic review and meta-analysis7
of clinical trials. J Clin Oncol 2010;28:2280–2285.

Chu, T. F., Rupnick, M. A., Kerkela, R., Dallabrida, S. M., Zurakowski, D., Nguyen, L., Woulfe, K., Pravda, E., Cassiola, F., and Desai, J. (2007) 'Cardiotoxicity Associated with Tyrosine Kinase Inhibitor Sunitinib'. *The Lancet* 370 (9604), 2011-2019.

Clarke M., Collins R., Darby S. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;366(9503):2087–2106.

Cohen, J., Babiarz, J., Abrams, R., Guo, L., Kameoka, S., Chiao, E., Taunton, J., and Kolaja, K. (2011) 'Use of Human Stem Cell Derived Cardiomyocytes to Examine Sunitinib Mediated Cardiotoxicity and Electrophysiological Alterations'*. Toxicology and Applied Pharmacology* 257 (1), 74-83.

Cooper, S. L., Sandhu, H., Hussain, A., Mee, C., and Maddock, H. (2018) 'Involvement of Mitogen Activated Kinase Kinase 7 Intracellular Signalling Pathway in Sunitinib-Induced Cardiotoxicity'*. Toxicology* 394, 72-83.

Corradetti, M. N., Inoki, K., Bardeesy, N., DePinho, R. A., and Guan, K. L. (2004) 'Regulation of the TSC Pathway by LKB1: Evidence of a Molecular Link between Tuberous Sclerosis Complex and Peutz-Jeghers Syndrome'*. Genes & Development* 18 (13), 1533-1538.

Corrado, D., Basso, C., Thiene, G., McKenna, W. J., Davies, M. J., Fontaliran, F., Nava, A., Silvestri, F., Blomstrom-Lundqvist, C., and Wlodarska, E. K. (1997) 'Spectrum of Clinicopathologic Manifestations of Arrhythmogenic Right Ventricular cardiomyopathy/dysplasia: A Multicenter Study'*. Journal of the American College of Cardiology* 30 (6), 1512-1520.

Corremans R, Vervaet BA, D'Haese PC, Neven E, Verhulst A. (2019) 'Metformin: A candidate drug for renal diseases'. *International journal of molecular sciences*, 20(1):42.

Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E., Salmon, M., and Lord, J. M. (2000) 'Serine/threonine Protein Kinases and Apoptosis'*. Experimental Cell Research* 256 (1), 34-41.

Crute, B. E., Seefeld, K., Gamble, J., Kemp, B. E., and Witters, L. A. (1998) 'Functional Domains of the alpha1 Catalytic Subunit of the AMP-Activated Protein Kinase'*. The Journal of Biological Chemistry* 273 (52), 35347-35354.

Curigliano, G., Cardinale, D., Dent, S., Criscitiello, C., Aseyev, O., Lenihan, D., and Cipolla, C. M. (2016) 'Cardiotoxicity of Anticancer Treatments: Epidemiology, Detection, and Management'. *CA: A Cancer Journal for Clinicians* 66 (4), 309-325.

Cusi, K. (1998) 'Metformin: A Review of its Metabolic Effects'. *Diabetes Reviews* 6, 89-131.

Dahlgren, E., Janson, P., Johansson, S., Lapidus, L., and Oden, A. (1992) 'Polycystic Ovary Syndrome and Risk for Myocardial Infarction: Evaluated from a Risk Factor Model Based on a Prospective Population Study of Women'. *Acta Obstetricia Et Gynecologica Scandinavica* 71 (8), 599-604.

Darby S., McGale P., Correa C. Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. Lancet. 2011;378(9804):1707–1716.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) 'Akt Phosphorylation of BAD Couples Survival Signals to the Cell-Intrinsic Death Machinery'. *Cell* 91 (2), 231-241.

de Boer, R. A., Doehner, W., van der Horst, Iwan CC, Anker, S. D., Babalis, D., Roughton, M., Coats, A. J., Flather, M. D., van Veldhuisen, D. J., and SENIORS Investigators (2010) 'Influence of Diabetes Mellitus and Hyperglycemia on Prognosis in Patients≥ 70 Years Old with Heart Failure and Effects of Nebivolol (Data from the Study of Effects of Nebivolol Intervention on Outcomes and Rehospitalization in Seniors with Heart Failure [SENIORS])'. *The American Journal of Cardiology* 106 (1), 78-86. e1.

DeFronzo, R. A., Tobin, J. D., and Andres, R. (1979) 'Glucose Clamp Technique: A Method for Quantifying Insulin Secretion and Resistance'. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 237 (3), G214-G223.

De Haas, E. C., Oosting, S. F., Lefrandt, J. D., Wolffenbuttel, B. H., Sleijfer, D. T., and Gietema, J. A. (2010) 'The Metabolic Syndrome in Cancer Survivors'. *The Lancet Oncology* 11 (2), 193-203.

Demetri, G. D., van Oosterom, A. T., Garrett, C. R., Blackstein, M. E., Shah, M. H., Verweij, J., McArthur, G., Judson, I. R., Heinrich, M. C., and Morgan, J. A. (2006) 'Efficacy and Safety of Sunitinib in Patients with Advanced Gastrointestinal Stromal Tumour After Failure of Imatinib: A Randomised Controlled Trial'. *The Lancet* 368 (9544), 1329-1338.

Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S. C., and Thomas, G. (2001) 'Mammalian TOR: A Homeostatic ATP Sensor'. *Science (New York, N.Y.)* 294 (5544), 1102-1105.

Detaille, D., Guigas, B., Chauvin, C., Batandier, C., Fontaine, E., Wiernsperger, N., and Leverve, X. (2005) 'Metformin Prevents High-Glucose-Induced Endothelial Cell Death through a Mitochondrial Permeability Transition-Dependent Process'. *Diabetes* 54 (7), 2179-2187.

DeYoung, M. P., Horak, P., Sofer, A., Sgroi, D., and Ellisen, L. W. (2008) 'Hypoxia Regulates TSC1/2-mTOR Signaling and Tumor Suppression through REDD1-Mediated 14-3-3 Shuttling'. *Genes & Development* 22 (2), 239-251.

Dezfulian, C., Raat, N., Shiva, S., and Gladwin, M. T. (2007) 'Role of the Anion Nitrite in Ischemia-Reperfusion Cytoprotection and Therapeutics'. *Cardiovascular Research* 75 (2), 327-338.

Diabetes (2015) Diabetes Life Expectancy. Diabetes.co.uk. [online] available from < http://www.diabetes.co.uk/diabetes-life-expectancy.html> [17 June 2015].

Diamanti-Kandarakis, E. and Papavassiliou, A. G. (2006) 'Molecular Mechanisms of Insulin Resistance in Polycystic Ovary Syndrome'. *Trends in Molecular Medicine* 12 (7), 324-332.

Doenst, T., Nguyen, T. D., and Abel, E. D. (2013) 'Cardiac Metabolism in Heart Failure: Implications Beyond ATP Production'. *Circulation Research* 113 (6), 709-724.

Dong, L., Zhou, Q., Zhang, Z., Zhu, Y., Duan, T., and Feng, Y. (2012) 'Metformin Sensitizes Endometrial Cancer Cells to Chemotherapy by Repressing Glyoxalase I Expression'. *Journal of Obstetrics and Gynaecology Research* 38 (8), 1077-1085.

Doring, H. J. (1990) 'The Isolated Perfused Heart According to Langendorff Technique--Function--Application'. *Physiologia Bohemoslovaca* 39 (6), 481-504.

Dowling, R. J., Zakikhani, M., Fantus, I. G., Pollak, M., and Sonenberg, N. (2007) 'Metformin Inhibits Mammalian Target of Rapamycin-Dependent Translation Initiation in Breast Cancer Cells'. *Cancer Research* 67 (22), 10804-10812.

Driver, C., Bamitale, K. D. S., Kazi, A., Olla, M., Nyane, N. A., and Owira, P. M. O. (2018) 'Cardioprotective Effects of Metformin'. *Journal of Cardiovascular Pharmacology* 72 (2), 121-127.

Dumitrescu, C., Biondi, R., Xia, Y., Cardounel, A. J., Druhan, L. J., Ambrosio, G., and Zweier, J. L. (2007) 'Myocardial Ischemia Results in Tetrahydrobiopterin (BH4) Oxidation with Impaired Endothelial Function Ameliorated by BH4'. *Proceedings of the National Academy of Sciences of the United States of America* 104 (38), 15081-15086.

Du Toit, E. F. and Donner, D. G. (2012) 'Myocardial Insulin Resistance: An Overview of its Causes, Effects, and Potential Therapy'. *Insulin Resistance*, 189.

Düvel, K., Yecies, J. L., Menon, S., Raman, P., Lipovsky, A. I., Souza, A. L., Triantafellow, E., Ma, Q., Gorski, R., and Cleaver, S. (2010) 'Activation of a Metabolic Gene Regulatory Network Downstream of mTOR Complex 1'. *Molecular Cell* 39 (2), 171-183.

Dyck, J. R., Kudo, N., Barr, A. J., Davies, S. P., Hardie, D. G., and Lopaschuk, G. D. (1999) 'Phosphorylation Control of Cardiac acetyl-CoA Carboxylase by cAMP-dependent Protein Kinase and 5′-AMP Activated Protein Kinase'. *The FEBS Journal* 262 (1), 184-190.

Dyck, J. R. and Lopaschuk, G. D. (2006) 'AMPK Alterations in Cardiac Physiology and Pathology: Enemy Or Ally?'. *The Journal of Physiology* 574 (1), 95-112.

Efeyan, A., Zoncu, R., Chang, S., Gumper, I., Snitkin, H., Wolfson, R. L., Kirak, O., Sabatini, D. D., and Sabatini, D. M. (2013) 'Regulation of mTORC1 by the Rag GTPases is Necessary for Neonatal Autophagy and Survival'. *Nature* 493 (7434), 679-683.

Egan, D., Kim, J., Shaw, R. J., and Guan, K. (2011) 'The Autophagy Initiating Kinase ULK1 is Regulated Via Opposing Phosphorylation by AMPK and mTOR'. *Autophagy.*

Ellis, L. M. and Hicklin, D. J. (2008) 'VEGF-Targeted Therapy: Mechanisms of Anti-Tumour Activity'. *Nature Reviews.Cancer* 8 (8), 579.

El Messaoudi, S., Schreuder, T. H., Kengen, R. D., Rongen, G. A., van den Broek, Petra H, Thijssen, D. H., and Riksen, N. P. (2014) 'Impact of Metformin on Endothelial Ischemia-Reperfusion Injury in Humans in Vivo: A Prospective Randomized Open, Blinded-Endpoint Study'. *PloS One* 9 (4), e96062.

El-Mir, M., Nogueira, V., Fontaine, E., Avéret, N., Rigoulet, M., and Leverve, X. (2000) 'Dimethylbiguanide Inhibits Cell Respiration Via an Indirect Effect Targeted on the Respiratory Chain Complex I'. *Journal of Biological Chemistry* 275 (1), 223-228.

El-Serag, H. B. and Mason, A. C. (1999) 'Rising Incidence of Hepatocellular Carcinoma in the United States'. *New England Journal of Medicine* 340 (10), 745-750.

Eltzschig, H. K. and Eckle, T. (2011) 'Ischemia and Reperfusion [Mdash] from Mechanism to Translation'. *Nature Medicine* 17 (11), 1391-1401.

Ely, S. W. and Berne, R. M. (1992) 'Protective Effects of Adenosine in Myocardial Ischemia'. *Circulation* 85 (3), 893-904.

Erbas, O. and Yilmaz, M. (2013) 'Metoprolol and Diltiazem Ameliorate Ziprasidone-Induced Prolonged Corrected QT Interval in Rats'. *Toxicology and Industrial Health*.

Eremina, V., Jefferson, J. A., Kowalewska, J., Hochster, H., Haas, M., Weisstuch, J., Richardson, C., Kopp, J. B., Kabir, M. G., and Backx, P. H. (2008) 'VEGF Inhibition and Renal Thrombotic Microangiopathy'. *New England Journal of Medicine* 358 (11), 1129-1136.

Erices, R., Bravo, M. L., Gonzalez, P., Oliva, B., Racordon, D., Garrido, M., Ibanez, C., Kato, S., Branes, J., Pizarro, J., Barriga, M. I., Barra, A., Bravo, E., Alonso, C.,

Bustamente, E., Cuello, M. A., and Owen, G. I. (2013) 'Metformin, at Concentrations Corresponding to the Treatment of Diabetes, Potentiates the Cytotoxic Effects of Carboplatin in Cultures of Ovarian Cancer Cells'*. Reproductive Sciences (Thousand Oaks, Calif.)* 20 (12), 1433-1446.

Eschenhagen, T., Force, T., Ewer, M. S., Keulenaer, G. W., Suter, T. M., Anker, S. D., Avkiran, M., Azambuja, E., Balligand, J., and Brutsaert, D. L. (2011) 'Cardiovascular Side Effects of Cancer Therapies: A Position Statement from the Heart Failure Association of the European Society of Cardiology'*. European Journal of Heart Failure* 13 (1), 1-10.

Escobar-Morreale, H., Pazos, F., Potau, N., Garcia-Robles, R., Sancho, J. M., and Varela, C. (1994) 'Ovarian Suppression with Triptorelin and Adrenal Stimulation with Adrenocorticotropin in Functional Hyperadrogenism: Role of Adrenal and Ovarian Cytochrome P450c17 Alpha'*. Fertility and Sterility* 62 (3), 521-530.

Escobar-Morreale, H. F., Serrano-Gotarredona, J., Garcia-Robles, R., Sancho, J., and Varela, C. (1997) 'Lack of an Ovarian Function Influence on the Increased Adrenal Androgen Secretion Present in Women with Functional Ovarian Hyperandrogenism'*. Fertility and Sterility* 67 (4), 654-662.

ESHRE, T. R. and Group, ASRM-Sponsored PCOS Consensus Workshop (2004) 'Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome'*. Fertility and Sterility* 81 (1), 19-25.

Evans, J. M., Donnelly, L. A., Emslie-Smith, A. M., Alessi, D. R., and Morris, A. D. (2005) 'Metformin and Reduced Risk of Cancer in Diabetic Patients'*. BMJ (Clinical Research Ed.)* 330 (7503), 1304-1305.

Ewer, M. S. and Ewer, S. M. (2010) 'Cardiotoxicity of Anticancer Treatments: What the Cardiologist Needs to Know'*. Nature Reviews Cardiology* 7 (10).

Fabian, M. A., Biggs, W. H., Treiber, D. K., Atteridge, C. E., Azimioara, M. D., Benedetti, M. G., Carter, T. A., Ciceri, P., Edeen, P. T., and Floyd, M. (2005) 'A Small molecule–kinase Interaction Map for Clinical Kinase Inhibitors'*. Nature Biotechnology* 23 (3), 329-336.

Faivre, S., Delbaldo, C., Vera, K., Robert, C., Lozahic, S., Lassau, N., Bello, C., Deprimo, S., Brega, N., Massimini, G., Armand, J. P., Scigalla, P., and Raymond, E. (2006) 'Safety, Pharmacokinetic, and Antitumor Activity of SU11248, a Novel Oral Multitarget Tyrosine Kinase Inhibitor, in Patients with Cancer'*. Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 24 (1), 25-35.

Faivre, S., Demetri, G., Sargent, W., and Raymond, E. (2007) 'Molecular Basis for Sunitinib Efficacy and Future Clinical Development'. *Nature Reviews Drug Discovery* 6 (9), 734.

FDA (nd) 'Metformin Hydrochloride Tablets' *U.S. Food and Drug Administration* [online] available from < http://www.fda.gov/ohrms/dockets/dailys/02/May02/053102/800471e6.pdf> [29th June 2015].

FDA (2013) 'FDA Drug Safety Communication: FDA requires removal of some prescribing and dispensing restrictions for rosiglitazone-containing diabetes medicines'. *U.S. Food and Drug Administration.* [Online] available from < http://www.fda.gov/Drugs/DrugSafety/ucm376389.html > [26th June 2015].

Ferreira, G. D., Germeyer, A., de Barros Machado, A., do Nascimento, T. L., Strowitzki, T., Brum, I. S., von Eye Corleta, H., and Capp, E. (2014) 'Metformin Modulates PI3K and GLUT4 Expression and Akt/PKB Phosphorylation in Human Endometrial Stromal Cells After Stimulation with Androgen and Insulin'*. European Journal of Obstetrics & Gynecology and Reproductive Biology* 175, 157-162.

Ferri, N., Siegl, P., Corsini, A., Herrmann, J., Lerman, A., and Benghozi, R. (2013) 'Drug Attrition during Pre-Clinical and Clinical Development: Understanding and Managing Drug-Induced Cardiotoxicity'*. Pharmacology & Therapeutics*.

Fischer, F., Hamann, A., and Osiewacz, H. D. (2012) 'Mitochondrial Quality Control: An Integrated Network of Pathways'*. Trends in Biochemical Sciences* 37 (7), 284-292.

Fliss, H. and Gattinger, D. (1996) 'Apoptosis in Ischemic and Reperfused Rat Myocardium'. *Circulation Research* 79 (5), 949-956.

Folkman, J. (2007) 'Opinion: Angiogenesis: An Organizing Principle for Drug Discovery?'. *Nature Reviews Drug Discovery* 6 (4), 273.

Force, T., Krause, D. S., and Van Etten, R. A. (2007) 'Molecular Mechanisms of Cardiotoxicity of Tyrosine Kinase Inhibition'. *Nature Reviews Cancer* 7 (5), 332-344.

Foretz, M., Hebrard, S., Leclerc, J., Zarrinpashneh, E., Soty, M., Mithieux, G., Sakamoto, K., Andreelli, F., and Viollet, B. (2010) 'Metformin Inhibits Hepatic Gluconeogenesis in Mice Independently of the LKB1/AMPK Pathway via a Decrease in Hepatic Energy State'. *The Journal of Clinical Investigation* 120 (7), 2355-2369.

Franco, O. H., Peeters, A., Bonneux, L., and de Laet, C. (2005) 'Blood Pressure in Adulthood and Life Expectancy with Cardiovascular Disease in Men and Women: Life Course Analysis'. *Hypertension* 46 (2), 280-286.

Frank, E., Nothmann, M., and Wagner, A. (1926) 'Über Synthetisch Dargestellte Körper Mit Insulinartiger Wirkung Auf Den Normalen Und Diabetischen Organismus'. *Journal of Molecular Medicine* 5 (45), 2100-2107.

Franks, S., Gilling-Smith, C., Gharani, N., and McCarthy, M. (2000) 'Pathogenesis of Polycystic Ovary Syndrome: Evidence for a Genetically Determined Disorder of Ovarian Androgen Production'. *Human Fertility* 3 (2), 77-79.

Frederich, M. and Balschi, J. A. (2002) 'The Relationship between AMP-Activated Protein Kinase Activity and AMP Concentration in the Isolated Perfused Rat Heart'. *The Journal of Biological Chemistry* 277 (3), 1928-1932.

Frederich, M., Zhang, L., and Balschi, J. A. (2005) 'Hypoxia and AMP Independently Regulate AMP-Activated Protein Kinase Activity in Heart'. *American Journal of Physiology-Heart and Circulatory Physiology* 288 (5), H2412-H2421.

Frey, N., Katus, H. A., Olson, E. N., and Hill, J. A. (2004) 'Hypertrophy of the Heart: A New Therapeutic Target?'. *Circulation* 109 (13), 1580-1589.

Fu, X., Wan, S., Lyu, Y. L., Liu, L. F., and Qi, H. (2008) 'Etoposide Induces ATM-Dependent Mitochondrial Biogenesis through AMPK Activation'. *PLoS One* 3 (4), e2009.

Fu, Y., Xiao, H., Ma, X., Jiang, S., Xu, M., and Zhang, Y. (2011) 'Metformin Attenuates Pressure Overload-Induced Cardiac Hypertrophy Via AMPK Activation'. *Acta Pharmacologica Sinica* 32 (7), 879.

Fujise, K., Zhang, D., Liu, J., and Yeh, E. T. (2000) 'Regulation of Apoptosis and Cell Cycle Progression by MCL1. Differential Role of Proliferating Cell Nuclear Antigen'. *The Journal of Biological Chemistry* 275 (50), 39458-39465.

Fujita, K., Maeda, N., Sonoda, M., Ohashi, K., Hibuse, T., Nishizawa, H., Nishida, M., Hiuge, A., Kurata, A., Kihara, S., Shimomura, I., and Funahashi, T. (2008) 'Adiponectin Protects Against Angiotensin II-Induced Cardiac Fibrosis through Activation of PPAR-Alpha'. *Arteriosclerosis, Thrombosis, and Vascular Biology* 28 (5), 863-870.

Furui, K., Suganuma, N., Tsukahara, S., Asada, Y., Kikkawa, F., Tanaka, M., Ozawa, T., and Tomoda, Y. (1994) 'Identification of Two Point Mutations in the Gene Coding Luteinizing Hormone (LH) Beta-Subunit, Associated with Immunologically Anomalous LH Variants'. *The Journal of Clinical Endocrinology and Metabolism* 78 (1), 107-113.

Grabinski, N., Ewald, F., Hofmann, B. T., Staufer, K., Schumacher, U., Nashan, B., and Jücker, M. (2012) 'Combined Targeting of AKT and mTOR Synergistically Inhibits Proliferation of Hepatocellular Carcinoma Cells'. *Molecular Cancer* 11 (1), 85.

Gao, G., Widmer, J., Stapleton, D., Teh, T., Cox, T., Kemp, B. E., and Witters, L. A. (1995) 'Catalytic Subunits of the Porcine and Rat 5′-AMP-Activated Protein Kinase are Members of the SNF1 Protein Kinase Family'. *Biochimica Et Biophysica Acta (BBA)-Molecular Cell Research* 1266 (1), 73-82.

Gao, X. and Pan, D. (2001) 'TSC1 and TSC2 Tumor Suppressors Antagonize Insulin Signaling in Cell Growth'. *Genes & Development* 15 (11), 1383-1392.

Ghatalia P, Morgan CJ, Je Y, Nguyen PL, Trinh QD, Choueiri TK, Sonpavde G. Congestive heart failure with vascular endothelial growth factor receptor tyrosine kinase inhibitors. Crit Rev Oncol Hematol 2015;94:228–237.

Ghavami, S., Mutawe, M. M., Sharma, P., Yeganeh, B., McNeill, K. D., Klonisch, T., Unruh, H., Kashani, H. H., Schaafsma, D., and Los, M. (2011) 'Mevalonate Cascade Regulation of Airway Mesenchymal Cell Autophagy and Apoptosis: A Dual Role for p53'*. PloS One* 6 (1), e16523.

Giannarelli, R., Aragona, M., Coppelli, A., and Del Prato, S. (2003) 'Reducing Insulin Resistance with Metformin: The Evidence Today'*. Diabetes & Metabolism* 29 (4), 6S28-6S35.

Giovannucci, E., Harlan, D. M., Archer, M. C., Bergenstal, R. M., Gapstur, S. M., Habel, L. A., Pollak, M., Regensteiner, J. G., and Yee, D. (2010) 'Diabetes and Cancer: A Consensus Report'*. CA: A Cancer Journal for Clinicians* 60 (4), 207-221.

Glassman, A. H. and Bigger Jr, J. T. (2001) 'Antipsychotic Drugs: Prolonged QTc Interval, Torsade De Pointes, and Sudden Death'*. American Journal of Psychiatry* 158 (11), 1774-1782.

Glueck, C., Fontaine, R., Wang, P., Subbiah, M., Weber, K., Illig, E., Streicher, P., Sieve-Smith, L., Tracy, T., and Lang, J. (2001) 'Metformin Reduces Weight, Centripetal Obesity, Insulin, Leptin, and Low-Density Lipoprotein Cholesterol in Nondiabetic, Morbidly Obese Subjects with Body Mass Index Greater than 30'*. Metabolism* 50 (7), 856-861.

Goede, V., Fischer, K., Busch, R., Engelke, A., Eichhorst, B., Wendtner, C. M., Chagorova, T., de la Serna, J., Dilhuydy, M., and Illmer, T. (2014) 'Obinutuzumab Plus Chlorambucil in Patients with CLL and Coexisting Conditions'*. New England Journal of Medicine* 370 (12), 1101-1110.

Gokcel, A., Gumurdulu, Y., Karakose, H., Melek Ertorer, E., Tanaci, N., Bascil Tutuncu, N., and Guvener, N. (2002) 'Evaluation of the Safety and Efficacy of Sibutramine, Orlistat and Metformin in the Treatment of Obesity'*. Diabetes, Obesity and Metabolism* 4 (1), 49-55.

Gonzalez-Angulo, A. M. and Meric-Bernstam, F. (2010) 'Metformin: A Therapeutic Opportunity in Breast Cancer'. *Clinical Cancer Research* 16 (6), 1695-1700.

Goodman, V. L., Rock, E. P., Dagher, R., Ramchandani, R. P., Abraham, S., Gobburu, J. V., Booth, B. P., Verbois, S. L., Morse, D. E., Liang, C. Y., Chidambaram, N., Jiang, J. X., Tang, S., Mahjoob, K., Justice, R., and Pazdur, R. (2007) 'Approval Summary: Sunitinib for the Treatment of Imatinib Refractory Or Intolerant Gastrointestinal Stromal Tumors and Advanced Renal Cell Carcinoma'. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 13 (5), 1367-1373.

Goodwin, G. W., Taylor, C. S., and Taegtmeyer, H. (1998) 'Regulation of Energy Metabolism of the Heart during Acute Increase in Heart Work'. *Journal of Biological Chemistry* 273 (45), 29530-29539.

Gorini, S., De Angelis, A., Berrino, L., Malara, N., Rosano, G., and Ferraro, E. (2018) 'Chemotherapeutic Drugs and Mitochondrial Dysfunction: Focus on Doxorubicin, Trastuzumab, and Sunitinib'. *Oxidative Medicine and Cellular Longevity* 2018.

Gorski, P. A., Ceholski, D. K., and Hajjar, R. J. (2015) 'Altered Myocardial Calcium Cycling and Energetics in Heart failure—a Rational Approach for Disease Treatment'. *Cell Metabolism* 21 (2), 183-194.

Gray, J. H., Owen, R. P., and Giacomini, K. M. (2004) 'The Concentrative Nucleoside Transporter Family, SLC28'. *Pflügers Archiv* 447 (5), 728-734.

Green, A. S., Chapuis, N., Maciel, T. T., Willems, L., Lambert, M., Arnoult, C., Boyer, O., Bardet, V., Park, S., Foretz, M., Viollet, B., Ifrah, N., Dreyfus, F., Hermine, O., Moura, I. C., Lacombe, C., Mayeux, P., Bouscary, D., and Tamburini, J. (2010) 'The LKB1/AMPK Signaling Pathway has Tumor Suppressor Activity in Acute Myeloid Leukemia through the Repression of mTOR-Dependent Oncogenic mRNA Translation'. *Blood* 116 (20), 4262-4273.

Green, A. S., Chapuis, N., Lacombe, C., Mayeux, P., Bouscary, D., and Tamburini, J. (2011) 'LKB1/AMPK/mTOR Signaling Pathway in Hematological Malignancies: From Metabolism to Cancer Cell Biology'. *Cell Cycle* 10 (13), 2115-2120.

Greineder, C. F., Kohnstamm, S., and Ky, B. (2011) 'Heart Failure Associated with Sunitinib: Lessons Learned from Animal Models'. *Current Hypertension Reports* 13 (6), 436-441.

Griffiths, E. J. and Halestrap, A. P. (1995) 'Mitochondrial Non-Specific Pores Remain Closed during Cardiac Ischaemia, but Open upon Reperfusion'. *Biochem.J* 307, 93-98.

Gundewar, S., Calvert, J. W., Jha, S., Toedt-Pingel, I., Ji, S. Y., Nunez, D., Ramachandran, A., Anaya-Cisneros, M., Tian, R., and Lefer, D. J. (2009) 'Activation of AMP-Activated Protein Kinase by Metformin Improves Left Ventricular Function and Survival in Heart Failure'. *Circulation Research* 104 (3), 403-411.

Gungor, N., Hannon, T., Libman, I., Bacha, F., and Arslanian, S. (2005) 'Type 2 Diabetes Mellitus in Youth: The Complete Picture to Date'. *Pediatric Clinics of North America* 52 (6), 1579-1609.

Gunton, J. E., Delhanty, P. J., Takahashi, S., and Baxter, R. C. (2003) 'Metformin Rapidly Increases Insulin Receptor Activation in Human Liver and Signals Preferentially through Insulin-Receptor Substrate-2'. *The Journal of Clinical Endocrinology & Metabolism* 88 (3), 1323-1332.

Guo, J., Wang, T., Yang, T., Xu, J., Li, W., Fridman, M. D., Fisher, J. T., and Zhang, S. (2011) 'Interaction between the Cardiac Rapidly (IKr) and Slowly (IKs) Activating Delayed Rectifier Potassium Channels Revealed by Low K+-Induced hERG Endocytic Degradation'. *The Journal of Biological Chemistry* 286 (40), 34664-34674.

Gruzdeva, O., Uchasova, E., Belik, E., Dyleva, Y., Shurygina, E., and Barbarash, O. (2014) 'Lipid, Adipokine and Ghrelin Levels in Myocardial Infarction Patients with Insulin Resistance'. *BMC Cardiovascular Disorders* 14, 7-2261-14-7.

Gschwind, A., Fischer, O. M., and Ullrich, A. (2004) 'The Discovery of Receptor Tyrosine Kinases: Targets for Cancer Therapy'. *Nature Reviews.Cancer* 4 (5), 361.

GSK (2014) ‘Avandia tablets’ *GSK Source* [online] available from <https://www.gsksource.com/gskprm/htdocs/documents/AVANDIA-PI-MG.PDF> [08/04/2015].

Guigas, B., Detaille, D., Chauvin, C., Batandier, C., De Oliveira, F., Fontaine, E., and Leverve, X. (2004) 'Metformin Inhibits Mitochondrial Permeability Transition and Cell Death: A Pharmacological in Vitro Study'*. Biochem.J*382, 877-884.

Gupta, A., Chacko, V. P., and Weiss, R. G. (2009) 'Abnormal Energetics and ATP Depletion in Pressure-Overload Mouse Hearts: In Vivo High-Energy Phosphate Concentration Measures by Noninvasive Magnetic Resonance'*. American Journal of Physiology-Heart and Circulatory Physiology* 297 (1), H59-H64.

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E., and Shaw, R. J. (2008) 'AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint'*. Molecular Cell* 30 (2), 214-226.

Hamieh, L., McKay, R. R., Lin, X., Moreira, R. B., Simantov, R., and Choueiri, T. K. (2017) 'Effect of Metformin use on Survival Outcomes in Patients with Metastatic Renal Cell Carcinoma'*. Clinical Genitourinary Cancer* 15 (2), 221-229.

Haffner, S. M., Lehto, S., Rönnemaa, T., Pyörälä, K., and Laakso, M. (1998) 'Mortality from Coronary Heart Disease in Subjects with Type 2 Diabetes and in Nondiabetic Subjects with and without Prior Myocardial Infarction'*. New England Journal of Medicine* 339 (4), 229-234.

Hall, P. S., Harshman, L. C., Srinivas, S., and Witteles, R. M. (2013) 'The Frequency and Severity of Cardiovascular Toxicity from Targeted Therapy in Advanced Renal Cell Carcinoma Patients'*. JACC: Heart Failure* 1 (1), 72-78.

Hara, K., Yonezawa, K., Weng, Q., Kozlowski, M. T., Belham, C., and Avruch, J. (1998) 'Amino Acid Sufficiency and mTOR Regulate p70 S6 Kinase and eIF-4E BP1 through a Common Effector Mechanism'*. Journal of Biological Chemistry* 273 (23), 14484-14494.

Hardie, D. G. and Carling, D. (1997) 'The AMP-activated Protein Kinase: Fuel Gauge of the Mammalian Cell?'*. European Journal of Biochemistry* 246 (2), 259-273.

Hardie, D. G. and Hawley, S. A. (2001) 'AMP-Activated Protein Kinase: The Energy Charge Hypothesis Revisited'*. Bioessays* 23 (12), 1112-1119.

Hardie, D. G. (1992) 'Regulation of Fatty Acid and Cholesterol Metabolism by the AMP-Activated Protein Kinase'*. Biochimica Et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* 1123 (3), 231-238.

Hardie, D. G. (2003) 'Minireview: The AMP-Activated Protein Kinase Cascade: The Key Sensor of Cellular Energy Status'*. Endocrinology* 144 (12), 5179-5183.

Hardie, D. G. (2006) 'Neither LKB1 nor AMPK are the Direct Targets of Metformin'*. Gastroenterology* 131 (3), 973.

Hardie, D. G. (2011) 'AMP-Activated Protein Kinase: An Energy Sensor that Regulates all Aspects of Cell Function'*. Genes & Development* 25 (18), 1895-1908.

Hardie, D. G., Ross, F. A., and Hawley, S. A. (2012) 'AMPK: A Nutrient and Energy Sensor that Maintains Energy Homeostasis'*. Nature Reviews Molecular Cell Biology* 13 (4), 251-262.

Harvey, P. A. and Leinwand, L. A. (2011) 'The Cell Biology of Disease: Cellular Mechanisms of Cardiomyopathy'*. The Journal of Cell Biology* 194 (3), 355-365.

Harvey, P. A. and Leinwand, L. A. (2015) 'Oestrogen Enhances Cardiotoxicity Induced by Sunitinib by Regulation of Drug Transport and Metabolism'*. Cardiovascular Research* 107 (1), 66-77.

Hasinoff, B. B., Patel, D., and O'Hara, K. A. (2008) 'Mechanisms of Myocyte Cytotoxicity Induced by the Multiple Receptor Tyrosine Kinase Inhibitor Sunitinib'*. Molecular Pharmacology* 74 (6), 1722-1728.

Hausenloy, D. J., Maddock, H. L., Baxter, G. F., and Yellon, D. M. (2002) 'Inhibiting Mitochondrial Permeability Transition Pore Opening: A New Paradigm for Myocardial Preconditioning?'*. Cardiovascular Research* 55 (3), 534-543.

Hausenloy, D. J. and Yellon, D. M. (2003) 'The Mitochondrial Permeability Transition Pore: Its Fundamental Role in Mediating Cell Death during Ischaemia and Reperfusion'*. Journal of Molecular and Cellular Cardiology* 35 (4), 339-341.

Hausenloy, D. J. and Yellon, D. M. (2004) 'New Directions for Protecting the Heart Against Ischaemia-Reperfusion Injury: Targeting the Reperfusion Injury Salvage Kinase (RISK)-Pathway'*. Cardiovascular Research* 61 (3), 448-460.

Hausenloy, D. J., Mocanu, M. M., and Yellon, D. M. (2004) 'Cross-Talk between the Survival Kinases during Early Reperfusion: Its Contribution to Ischemic Preconditioning'*. Cardiovascular Research* 63 (2), 305-312.

Hausenloy, D. J., Bhamra, G. S., Davidson, S. M., Carr, R., Mocanu, M. M., and Yellon, D. M. (2007) 'Metformin Cardioprotects the Diabetic Heart by Inhibiting mPTP Opening Via the Risk Pathway'*. Journal of Molecular and Cellular Cardiology* 42 (6), S217.

Hausenloy, D. J. and Yellon, D. M. (2013) 'Myocardial Ischemia-Reperfusion Injury: A Neglected Therapeutic Target'*. The Journal of Clinical Investigation*123 (1), 92-100.

Hawley, S. A., Selbert, M. A., Goldstein, E. G., Edelman, A. M., Carling, D., and Hardie, D. G. (1995) '5'-AMP Activates the AMP-Activated Protein Kinase Cascade, and Ca2+/calmodulin Activates the Calmodulin-Dependent Protein Kinase I Cascade, Via Three Independent Mechanisms'*. The Journal of Biological Chemistry* 270 (45), 27186-27191.

Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Mäkelä, T. P., Alessi, D. R., and Hardie, D. G. (2003) 'Complexes between the LKB1 Tumor Suppressor, STRADα/β and MO25α/β are Upstream Kinases in the AMP-Activated Protein Kinase Cascade'*. Journal of Biology* 2 (4), 28.

Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., and Hardie, D. G. (2005) 'Calmodulin-Dependent Protein Kinase Kinase-β is an Alternative Upstream Kinase for AMP-Activated Protein Kinase'. *Cell Metabolism* 2 (1), 9-19.

Hawley, S. A., Ross, F. A., Chevtzoff, C., Green, K. A., Evans, A., Fogarty, S., Towler, M. C., Brown, L. J., Ogunbayo, O. A., and Evans, A. M. (2010) 'Use of Cells Expressing γ Subunit Variants to Identify Diverse Mechanisms of AMPK Activation'. *Cell Metabolism* 11 (6), 554-565.

Hay, N. and Sonenberg, N. (2004) 'Upstream and Downstream of mTOR'. *Genes & Development* 18 (16), 1926-1945.

He, C. and Klionsky, D. J. (2009) 'Regulation Mechanisms and Signaling Pathways of Autophagy'. *Annual Review of Genetics* 43.

Headrick, J. P., Hack, B., and Ashton, K. J. (2003) 'Acute Adenosinergic Cardioprotection in Ischemic-Reperfused Hearts'. *American Journal of Physiology.Heart and Circulatory Physiology* 285 (5), H1797-818. .

Heidrich, F., Schotola, H., Popov, A. F., Sohns, C., Schuenemann, J., Friedrich, M., Coskun, K. O., von Lewinski, D., Hinz, J., Bauer, M., Mokashi, S. A., Sossalla, S., and Schmitto, J. D. (2010) 'AMPK - Activated Protein Kinase and its Role in Energy Metabolism of the Heart'. *Current Cardiology Reviews* 6 (4), 337-342.

Heinze, T., Jonas, S., Karsten, A., and Neuhaus, P. (1999) 'Determination of the Oncogenes p53 and C-Erb B2 in the Tumour Cytosols of Advanced Hepatocellular Carcinoma (HCC) and Correlation to Survival Time'. *Anticancer Research* 19 (4A), 2501-2503.

Henderson, K. A., Borders, R. B., Ross, J. B., Huwar, T. B., Travis, C. O., Wood, B. J., Ma, Z. J., Hong, S. P., Vinci, T. M., and Roche, B. M. (2013) 'Effects of Tyrosine Kinase Inhibitors on Rat Isolated Heart Function and Protein Biomarkers Indicative of Toxicity'. *Journal of Pharmacological and Toxicological Methods* 68 (1), 150-159.

Hickson-Bick, D. L., Buja, M. L., and McMillin, J. B. (2000) 'Palmitate-Mediated Alterations in the Fatty Acid Metabolism of Rat Neonatal Cardiac Myocytes'. *Journal of Molecular and Cellular Cardiology* 32 (3), 511-519.

Hirsch, H. A., Iliopoulos, D., Tsichlis, P. N., and Struhl, K. (2009) 'Metformin Selectively Targets Cancer Stem Cells, and Acts Together with Chemotherapy to Block Tumor Growth and Prolong Remission'. *Cancer Research* 69 (19), 7507-7511.

Hirsch, H. A., Iliopoulos, D., and Struhl, K. (2013) 'Metformin Inhibits the Inflammatory Response Associated with Cellular Transformation and Cancer Stem Cell Growth'. *Proceedings of the National Academy of Sciences of the United States of America*110 (3), 972-977.

Hodgson D.C. Late effects in the era of modern therapy for Hodgkin lymphoma. Hematol Am Soc Hematol Educ Prog. 2011;2011:323–329.

Holman, R. R., Paul, S. K., Bethel, M. A., Matthews, D. R., and Neil, H. A. W. (2008) '10-Year Follow-Up of Intensive Glucose Control in Type 2 Diabetes'. *New England Journal of Medicine* 359 (15), 1577-1589.

Horman, S., Browne, G. J., Krause, U., Patel, J. V., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C. G., and Rider, M. H. (2002) 'Activation of AMP-Activated Protein Kinase Leads to the Phosphorylation of Elongation Factor 2 and an Inhibition of Protein Synthesis'. *Current Biology* 12 (16), 1419-1423.

Horman, S., Vertommen, D., Heath, R., Neumann, D., Mouton, V., Woods, A., Schlattner, U., Wallimann, T., Carling, D., Hue, L., and Rider, M. H. (2006) 'Insulin Antagonizes Ischemia-Induced Thr172 Phosphorylation of AMP-Activated Protein Kinase Alpha-Subunits in Heart Via Hierarchical Phosphorylation of Ser485/491'. *The Journal of Biological Chemistry* 281 (9), 5335-5340.

Hotamisligil, G. S., Shargill, N. S., and Spiegelman, B. M. (1993) 'Adipose Expression of Tumor Necrosis Factor-Alpha: Direct Role in Obesity-Linked Insulin Resistance'. *Science (New York, N.Y.)*259 (5091), 87-91.

Hsieh, P. C., MacGillivray, C., Gannon, J., Cruz, F. U., and Lee, R. T. (2006) 'Local Controlled Intramyocardial Delivery of Platelet-Derived Growth Factor Improves Postinfarction Ventricular Function without Pulmonary Toxicity'*. Circulation* 114 (7), 637-644.

Hu, Y., Liu, J., and Huang, H. (2013) 'Recent Agents Targeting HIF-1α for Cancer Therapy'*. Journal of Cellular Biochemistry* 114 (3), 498-509.

Huang, C. C., Wu, M. C., Xu, G. W., Li, D. Z., Cheng, H., Tu, Z. X., Jiang, H. Q., and Gu, J. R. (1992) 'Overexpression of the MDR1 Gene and P-Glycoprotein in Human Hepatocellular Carcinoma'*. Journal of the National Cancer Institute* 84 (4), 262-264.

Huang, X., Wullschleger, S., Shpiro, N., McGuire, V., Sakamoto, K., Woods, Y., McBurnie, W., Fleming, S., and Alessi, D. (2008) 'Important Role of the LKB1-AMPK Pathway in Suppressing Tumorigenesis in PTEN-Deficient Mice'*. Biochem.J* 412, 211-221.

Hudson, E. R., Pan, D. A., James, J., Lucocq, J. M., Hawley, S. A., Green, K. A., Baba, O., Terashima, T., and Hardie, D. G. (2003) 'A Novel Domain in AMP-Activated Protein Kinase Causes Glycogen Storage Bodies Similar to those seen in Hereditary Cardiac Arrhythmias'*. Current Biology* 13 (10), 861-866.

Huffman, J. C. and Stern, T. A. (2003) 'QTc Prolongation and the use of Antipsychotics: A Case Discussion'*. Primary Care Companion to the Journal of Clinical Psychiatry* 5 (6), 278-281.

Hurst, R. T. and Lee, R. W. (2003) 'Increased Incidence of Coronary Atherosclerosis in Type 2 Diabetes Mellitus: Mechanisms and Management'*. Annals of Internal Medicine* 139 (10), 824-834.

Huss, J. M. and Kelly, D. P. (2004) 'Nuclear Receptor Signaling and Cardiac Energetics'*. Circulation Research* 95 (6), 568-578.

Igata, M., Motoshima, H., Tsuruzoe, K., Kojima, K., Matsumura, T., Kondo, T., Taguchi, T., Nakamaru, K., Yano, M., Kukidome, D., Matsumoto, K., Toyonaga, T., Asano, T., Nishikawa, T., and Araki, E. (2005) 'Adenosine Monophosphate-

Activated Protein Kinase Suppresses Vascular Smooth Muscle Cell Proliferation through the Inhibition of Cell Cycle Progression'. *Circulation Research* 97 (8), 837-844.

Iliopoulos, D., Hirsch, H. A., and Struhl, K. (2011) 'Metformin Decreases the Dose of Chemotherapy for Prolonging Tumor Remission in Mouse Xenografts Involving Multiple Cancer Cell Types'. *Cancer Research* 71 (9), 3196-3201.

Ingwall, J. S. (1993) 'Adaptive and Maladaptive Processes: Is Cardiac Failure a Consequence of Decreased Energy Reserve?'. *Circulation* 87 (6S), VII58-VII62.

Ingwall, J. S. (2004) 'Transgenesis and Cardiac Energetics: New Insights into Cardiac Metabolism'. *Journal of Molecular and Cellular Cardiology* 37 (3), 613-623.

Ingwall, J. S. (2008) 'Energy Metabolism in Heart Failure and Remodelling'. *Cardiovascular Research* 81 (3), 412-419,

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. (2002) 'TSC2 is Phosphorylated and Inhibited by Akt and Suppresses mTOR Signalling'. *Nature Cell Biology* 4 (9), 648-657.

Inoki, K., Zhu, T., and Guan, K. (2003) 'TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival'. *Cell* 115 (5), 577-590.

Inoki, K., Ouyang, H., Li, Y., and Guan, K. L. (2005) 'Signaling by Target of Rapamycin Proteins in Cell Growth Control'. *Microbiology and Molecular Biology Reviews: MMBR* 69 (1), 79-100.

Inserte, J., Garcia-Dorado, D., Ruiz-Meana, M., Padilla, F., Barrabes, J. A., Pina, P., Agullo, L., Piper, H. M., and Soler-Soler, J. (2002) 'Effect of Inhibition of Na(+)/Ca(2+) Exchanger at the Time of Myocardial Reperfusion on Hypercontracture and Cell Death'. *Cardiovascular Research* 55 (4), 739-748.

International Agency for Research on Cancer (2012). *GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012.*

Inzucchi, S. E., Maggs, D. G., Spollett, G. R., Page, S. L., Rife, F. S., Walton, V., and Shulman, G. I. (1998) 'Efficacy and Metabolic Effects of Metformin and Troglitazone in Type II Diabetes Mellitus'*. New England Journal of Medicine* 338 (13), 867-873.

Isakovic, A., Harhaji, L., Stevanovic, D., Markovic, Z., Sumarac-Dumanovic, M., Starcevic, V., Micic, D., and Trajkovic, V. (2007) 'Dual Antiglioma Action of Metformin: Cell Cycle Arrest and Mitochondria-Dependent Apoptosis'*. Cellular and Molecular Life Sciences* 64 (10), 1290.

Ishihara, M., Inoue, I., Kawagoe, T., Shimatani, Y., Kurisu, S., Nakama, Y., Kagawa, E., Dai, K., Ootani, T., and Ikenaga, H. (2011) 'Glucometabolic Responses during Glucose Tolerance Test: A Comparison between Known Diabetes and Newly Detected Diabetes After Acute Myocardial Infarction'*. International Journal of Cardiology* 152 (1), 78-82.

Ismail, S., Mayah, W., Battia, H. E., Gaballah, H., Jiman-Fatani, A., Hamouda, H., Afifi, M. A., Elmashad, N., and Saadany, S. E. (2015) 'Plasma Nuclear Factor Kappa B and Serum Peroxiredoxin 3 in Early Diagnosis of Hepatocellular Carcinoma'*. Asian Pac J Cancer Prev* 16 (4), 1657-1663.

Ito, Y., Fisher, C. R., Conte, F. A., Grumbach, M. M., and Simpson, E. R. (1993) 'Molecular Basis of Aromatase Deficiency in an Adult Female with Sexual Infantilism and Polycystic Ovaries'*. Proceedings of the National Academy of Sciences of the United States of America* 90 (24), 11673-11677.

Jager, S., Handschin, C., St-Pierre, J., and Spiegelman, B. M. (2007) 'AMP-Activated Protein Kinase (AMPK) Action in Skeletal Muscle Via Direct Phosphorylation of PGC-1alpha'*. Proceedings of the National Academy of Sciences of the United States of America* 104 (29), 12017-12022.

Jain, R. K., Duda, D. G., Clark, J. W., and Loeffler, J. S. (2006) 'Lessons from Phase III Clinical Trials on Anti-VEGF Therapy for Cancer'*. Nature Clinical Practice Oncology* 3 (1), 24-40.

Jang, S. Y., Kim, A., Kim, J. K., Kim, C., Cho, Y. H., Kim, J. H., Kim, C. H., and Lee, J. Y. (2014) 'Metformin Inhibits Tumor Cell Migration Via Down-Regulation of MMP9

in Tamoxifen-Resistant Breast Cancer Cells'. *Anticancer Research* 34 (8), 4127-4134.

Janjetovic, K., Vucicevic, L., Misirkic, M., Vilimanovich, U., Tovilovic, G., Zogovic, N., Nikolic, Z., Jovanovic, S., Bumbasirevic, V., and Trajkovic, V. (2011) 'Metformin Reduces Cisplatin-Mediated Apoptotic Death of Cancer Cells through AMPK-Independent Activation of Akt'. *European Journal of Pharmacology* 651 (1), 41-50.

Javadov, S. A., Clarke, S., Das, M., Griffiths, E. J., Lim, K. H., and Halestrap, A. P. (2003) 'Ischaemic Preconditioning Inhibits Opening of Mitochondrial Permeability Transition Pores in the Reperfused Rat Heart'. *The Journal of Physiology* 549 (2), 513-524.

Jeon, S., Chandel, N. S., and Hay, N. (2012) 'AMPK Regulates NADPH Homeostasis to Promote Tumour Cell Survival during Energy Stress'. *Nature* 485 (7400), 661-665.

Jessen, N., Koh, H., Folmes, C. D., Wagg, C., Fujii, N., Løfgren, B., Wolf, C. M., Berul, C. I., Hirshman, M. F., and Lopaschuk, G. D. (2010) 'Ablation of LKB1 in the Heart Leads to Energy Deprivation and Impaired Cardiac Function'. *Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease* 1802 (7), 593-600.

Jin, J., Mullen, T. D., Hou, Q., Bielawski, J., Bielawska, A., Zhang, X., Obeid, L. M., Hannun, Y. A., and Hsu, Y. T. (2009) 'AMPK Inhibitor Compound C Stimulates Ceramide Production and Promotes Bax Redistribution and Apoptosis in MCF7 Breast Carcinoma Cells'. *Journal of Lipid Research* 50 (12), 2389-2397.

Jiralerspong, S., Palla, S. L., Giordano, S. H., Meric-Bernstam, F., Liedtke, C., Barnett, C. M., Hsu, L., Hung, M. C., Hortobagyi, G. N., and Gonzalez-Angulo, A. M. (2009) 'Metformin and Pathologic Complete Responses to Neoadjuvant Chemotherapy in Diabetic Patients with Breast Cancer'. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 27 (20), 3297-3302.

Johnson, J. A., Majumdar, S. R., Simpson, S. H., and Toth, E. L. (2002) 'Decreased Mortality Associated with the use of Metformin Compared with Sulfonylurea Monotherapy in Type 2 Diabetes'. *Diabetes Care* 25 (12), 2244-2248.

Jones, R. G., Plas, D. R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M. J., and Thompson, C. B. (2005) 'AMP-Activated Protein Kinase Induces a p53-Dependent Metabolic Checkpoint'. *Molecular Cell* 18 (3), 283-293.

Jones, R. G. and Thompson, C. B. (2009) 'Tumor Suppressors and Cell Metabolism: A Recipe for Cancer Growth'. *Genes & Development* 23 (5), 537-548.

Jung, E. J., Kwon, S. W., Jung, B. H., Oh, S. H., and Lee, B. H. (2011) 'Role of the AMPK/SREBP-1 Pathway in the Development of Orotic Acid-Induced Fatty Liver'. *Journal of Lipid Research* 52 (9), 1617-1625.

Junien, C. and McBride, W. (1988) 'Report of the Committee on the Genetic Constitution of Chromosome 11'. *Cytogenetic and Genome Research* 49 (1-3), 79-81.

Kakimoto, Y., Tsuruyama, T., Miyao, M., Abiru, H., Sumiyoshi, S., Kotani, H., Haga, H., and Tamaki, K. (2013) 'The Effectiveness and Limitations of Triphenyltetrazolium Chloride to Detect Acute Myocardial Infarction at Forensic Autopsy'. *The American Journal of Forensic Medicine and Pathology* 34 (3), 242-247.

Kalender, A., Selvaraj, A., Kim, S. Y., Gulati, P., Brûlé, S., Viollet, B., Kemp, B. E., Bardeesy, N., Dennis, P., and Schlager, J. J. (2010) 'Metformin, Independent of AMPK, Inhibits mTORC1 in a Rag GTPase-Dependent Manner'. *Cell Metabolism* 11 (5), 390-401.

Kamba, T. and McDonald, D. M. (2007) 'Mechanisms of Adverse Effects of Anti-VEGF Therapy for Cancer'. *British Journal of Cancer* 96 (12), 1788-1795.

Kappers, M. H., van Esch, J. H., Sluiter, W., Sleijfer, S., Danser, A. H., and van den Meiracker, A. H. (2010) 'Hypertension Induced by the Tyrosine Kinase Inhibitor Sunitinib is Associated with Increased Circulating Endothelin-1 Levels'. *Hypertension (Dallas, Tex.: 1979)* 56 (4), 675-681.

Kappers, M. H., Smedts, F. M., Horn, T., van Esch, J. H., Sleijfer, S., Leijten, F., Wesseling, S., Strevens, H., Jan Danser, A. H., and van den Meiracker, A. H. (2011) 'The Vascular Endothelial Growth Factor Receptor Inhibitor Sunitinib Causes a

Preeclampsia-Like Syndrome with Activation of the Endothelin System'. *Hypertension (Dallas, Tex.: 1979)* 58 (2), 295-302.

Karaman, M. W., Herrgard, S., Treiber, D. K., Gallant, P., Atteridge, C. E., Campbell, B. T., Chan, K. W., Ciceri, P., Davis, M. I., and Edeen, P. T. (2008) 'A Quantitative Analysis of Kinase Inhibitor Selectivity'. *Nature Biotechnology* 26 (1), 127-132.

Karrowni, W., Li, Y., Jones, P. G., Cresci, S., Abdallah, M. S., Lanfear, D. E., Maddox, T. M., McGuire, D. K., Spertus, J. A., and Horwitz, P. A. (2013) 'Insulin Resistance is Associated with Significant Clinical Atherosclerosis in Nondiabetic Patients with Acute Myocardial Infarction'. *Arteriosclerosis, Thrombosis, and Vascular Biology* 33 (9), 2245-2251.

Kawabata, H. and Ishikawa, K. (2003) 'Cardioprotection by Metformin is Abolished by a Nitric Oxide Synthase Inhibitor in Ischemic Rabbit Hearts.'. *Hypertension Research* 26 (1), 107-110.

Kawanaka, K., Nolte, L. A., Han, D. H., Hansen, P. A., and Holloszy, J. O. (2000) 'Mechanisms Underlying Impaired GLUT-4 Translocation in Glycogen-Supercompensated Muscles of Exercised Rats'. *American Journal of Physiology.Endocrinology and Metabolism* 279 (6), E1311-8.

Keizman, D., Ish-Shalom, M., Sella, A., Gottfried, M., Maimon, N., Peer, A., Hammers, H., Eisenberger, M. A., Sinibaldi, V., Neiman, V., Rosenbaum, E., Sarid, D., Mermershtain, W., Rouvinov, K., Berger, R., and Carducci, M. A. (2016) 'Metformin use and Outcome of Sunitinib Treatment in Patients with Diabetes and Metastatic Renal Cell Carcinoma'. *Clinical Genitourinary Cancer* 14 (5), 420-425.

Kelly, D. P. and Scarpulla, R. C. (2004) 'Transcriptional Regulatory Circuits Controlling Mitochondrial Biogenesis and Function'. *Genes & Development* 18 (4), 357-368.

Kerkelä, R., Grazette, L., Yacobi, R., Iliescu, C., Patten, R., Beahm, C., Walters, B., Shevtsov, S., Pesant, S., and Clubb, F. J. (2006) 'Cardiotoxicity of the Cancer Therapeutic Agent Imatinib Mesylate'. *Nature Medicine* 12 (8), 908.

Kerkela, R., Woulfe, K. C., Durand, J., Vagnozzi, R., Kramer, D., Chu, T. F., Beahm, C., Chen, M. H., and Force, T. (2009) 'Sunitinib-induced Cardiotoxicity is Mediated

by off-target Inhibition of AMP-activated Protein Kinase'. *Clinical and Translational Science* 2 (1), 15-25.

Kermani, A. and Garg, A. (eds.) (2003) *Mayo Clinic Proceedings*. 'Thiazolidinedione-Associated Congestive Heart Failure and Pulmonary Edema': Elsevier.

Kiiskinen, U., Vartiainen, E., Puska, P., and Aromaa, A. (1998) 'Long-term Cost and life-expectancy Consequences of Hypertension'. *Journal of Hypertension* 16 (8), 1103-1112.

Kim, J. S., Jin, Y., and Lemasters, J. J. (2006) 'Reactive Oxygen Species, but Not Ca2+ Overloading, Trigger pH- and Mitochondrial Permeability Transition-Dependent Death of Adult Rat Myocytes After Ischemia-Reperfusion'. *American Journal of Physiology.Heart and Circulatory Physiology* 290 (5), H2024-34.

Kim, M. H., Kim, M. O., Heo, J. S., Kim, J. S., and Han, H. J. (2008) 'Acetylcholine Inhibits Long-Term Hypoxia-Induced Apoptosis by Suppressing the Oxidative Stress-Mediated MAPKs Activation as Well as Regulation of Bcl-2, c-IAPs, and Caspase-3 in Mouse Embryonic Stem Cells'. *Apoptosis* 13 (2), 295-304.

King, H., Aubert, R. E., and Herman, W. H. (1998) 'Global Burden of Diabetes, 1995-2025: Prevalence, Numerical Estimates, and Projections'. *Diabetes Care* 21 (9), 1414-1431.

Kirpichnikov, D., McFarlane, S. I., and Sowers, J. R. (2002) 'Metformin: An Update'. *Annals of Internal Medicine*137 (1), 25-33.

Kita, Y., Takamura, T., Misu, H., Ota, T., Kurita, S., Takeshita, Y., Uno, M., Matsuzawa-Nagata, N., Kato, K., and Ando, H. (2012) 'Metformin Prevents and Reverses Inflammation in a Non-Diabetic Mouse Model of Nonalcoholic Steatohepatitis'.

Kjekshus, J. K., Torp-Pedersen, C., Gullestad, L., Køber, L., Edvardsen, T., Olsen, I. C., Sjaastad, I., Qvigstad, E., Skomedal, T., and Osnes, J. (2009) 'Effect of Piboserod, a 5-HT4 Serotonin Receptor Antagonist, on Left Ventricular Function in Patients with Symptomatic Heart Failure'. *European Journal of Heart Failure* 11 (8), 771-778.

Klip, A. and Leiter, L. A. (1990) 'Cellular Mechanism of Action of Metformin'. *Diabetes Care* 13 (6), 696-704.

Kloner, R. A., Ganote, C. E., and Jennings, R. B. (1974) 'The "no-Reflow" Phenomenon After Temporary Coronary Occlusion in the Dog'. *The Journal of Clinical Investigation* 54 (6), 1496-1508.

Krause, D. S. and Van Etten, R. A. (2005) 'Tyrosine Kinases as Targets for Cancer Therapy'. *New England Journal of Medicine* 353 (2), 172-187.

Kravchuk, E., Grineva, E., Bairamov, A., Galagudza, M., and Vlasov, T. (2011) 'The Effect of Metformin on the Myocardial Tolerance to Ischemia-Reperfusion Injury in the Rat Model of Diabetes Mellitus Type II'. *Experimental Diabetes Research* 2011, 907496.

Krentz, A., Viljoen, A., and Sinclair, A. (2013) 'Insulin Resistance: A Risk Marker for Disease and Disability in the Older Person'. *Diabetic Medicine* 30 (5), 535-548.

Kudo, N., Barr, A. J., Barr, R. L., Desai, S., and Lopaschuk, G. D. (1995) 'High Rates of Fatty Acid Oxidation during Reperfusion of Ischemic Hearts are Associated with a Decrease in Malonyl-CoA Levels due to an Increase in 5′-AMP-Activated Protein Kinase Inhibition of Acetyl-CoA Carboxylase'. *Journal of Biological Chemistry* 270 (29), 17513-17520.

Kudo, N., Gillespie, J. G., Kung, L., Witters, L. A., Schulz, R., Clanachan, A. S., and Lopaschuk, G. D. (1996) 'Characterization of 5′ AMP-Activated Protein Kinase Activity in the Heart and its Role in Inhibiting Acetyl-CoA Carboxylase during Reperfusion Following Ischemia'. *Biochimica Et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* 1301 (1-2), 67-75.

Kukidome, D., Nishikawa, T., Sonoda, K., Imoto, K., Fujisawa, K., Yano, M., Motoshima, H., Taguchi, T., Matsumura, T., and Araki, E. (2006) 'Activation of AMP-Activated Protein Kinase Reduces Hyperglycemia-Induced Mitochondrial Reactive Oxygen Species Production and Promotes Mitochondrial Biogenesis in Human Umbilical Vein Endothelial Cells'. *Diabetes* 55 (1), 120-127.

Laderoute, K., Calaoagan, J. M., Madrid, P. B., Klon, A. E., and Ehrlich, P. J. (2010) 'SU11248 (Sunitinib) Directly Inhibits the Activity of Mammalian 5'-AMP-Activated Protein Kinase (AMPK)'. *Cancer Biology & Therapy* 10 (1), 68-76.

Langendorff, O. (1898) 'Untersuchungen Am Überlebenden Säugethierherzen'. *Archiv Für Die Gesamte Physiologie Des Menschen Und Der Tiere* 70 (9-10), 473-486.

Lankheet, N. A., Blank, C. U., Mallo, H., Adriaansz, S., Rosing, H., Schellens, J. H., Huitema, A. D., and Beijnen, J. H. (2011) 'Determination of Sunitinib and its Active Metabolite N-Desethylsunitinib in Sweat of a Patient'. *Journal of Analytical Toxicology* 35 (8), 558-565.

Lankhorst, S., Kappers, M. H., van Esch, J. H., Danser, A. H., and van den Meiracker, A. H. (2013) 'Mechanism of Hypertension and Proteinuria during Angiogenesis Inhibition: Evolving Role of Endothelin-1'. *Journal of Hypertension* 31 (3), 444-54; discussion 454.

Lankhorst, S., Kappers, M. H., van Esch, J. H., Danser, A. J., and van den Meiracker, Anton H (2014) 'Hypertension during Vascular Endothelial Growth Factor Inhibition: Focus on Nitric Oxide, Endothelin-1, and Oxidative Stress'. *Antioxidants & Redox Signaling* 20 (1), 135-145.

Lankhorst, S., Baelde, H. J., Kappers, M. H., Smedts, F. M., Hansen, A., Clahsen-van Groningen, M. C., Sleijfer, S., Mathijssen, R. H., Danser, A. H., and van den Meiracker, A. H. (2015) 'Greater Sensitivity of Blood Pressure than Renal Toxicity to Tyrosine Kinase Receptor Inhibition with Sunitinib'. *Hypertension (Dallas, Tex.: 1979)* 66 (3), 543-549.

Larsson, S. C., Orsini, N., and Wolk, A. (2005) 'Diabetes Mellitus and Risk of Colorectal Cancer: A Meta-Analysis'. *Journal of the National Cancer Institute* 97 (22), 1679-1687.

Larsson, S. C., Mantzoros, C. S., and Wolk, A. (2007) 'Diabetes Mellitus and Risk of Breast Cancer: A meta-analysis'. *International Journal of Cancer* 121 (4), 856-862.

Lavin, F. M., and Khanna, K. K. (1999) 'ATM: The Protein Encoded by the Gene Mutated in the Radiosensitive Syndrome Ataxia-Telangiectasia'. *International Journal of Radiation Biology* 75 (10), 1201-1214.

Lazzeri, C., Sori, A., Chiostri, M., Gensini, G. F., and Valente, S. (2009) 'Prognostic Role of Insulin Resistance as Assessed by Homeostatic Model Assessment Index in the Acute Phase of Myocardial Infarction in Nondiabetic Patients Submitted to Percutaneous Coronary Intervention'. *European Journal of Anaesthesiology* 26 (10), 856-862.

L'Ecuyer, T., Horenstein, M. S., Thomas, R., and Vander Heide, R. (2001) 'Anthracycline-Induced Cardiac Injury using a Cardiac Cell Line: Potential for Gene Therapy Studies'. *Molecular Genetics and Metabolism* 74 (3), 370-379.

Lebovitz, H. E. (2001) 'Insulin Resistance: Definition and Consequences'. *Experimental and Clinical Endocrinology & Diabetes: Official Journal, German Society of Endocrinology [and] German Diabetes Association* 109 Suppl 2, S135-48.

Lee, J. O., Lee, S. K., Jung, J. H., Kim, J. H., You, G. Y., Kim, S. J., Park, S. H., Uhm, K., and Kim, H. S. (2011) 'Metformin Induces Rab4 through AMPK and Modulates GLUT4 Translocation in Skeletal Muscle Cells'. *Journal of Cellular Physiology* 226 (4), 974-981.

Lega, I. C., Austin, P. C., Gruneir, A., Goodwin, P. J., Rochon, P. A., and Lipscombe, L. L. (2013) 'Association between Metformin Therapy and Mortality After Breast Cancer: A Population-Based Study'. *Diabetes Care* 36 (10), 3018-3026.

Legtenberg, R. J., Houston, R. J., Oeseburg, B., and Smits, P. (2002) 'Metformin Improves Cardiac Functional Recovery After Ischemia in Rats'. *Hormone and Metabolic Research* 34 (04), 182-185.

Lehman, J. J., Barger, P. M., Kovacs, A., Saffitz, J. E., Medeiros, D. M., and Kelly, D. P. (2000) 'Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 Promotes Cardiac Mitochondrial Biogenesis'. *The Journal of Clinical Investigation* 106 (7), 847-856.

Leon, H., Atkinson, L. L., Sawicka, J., Strynadka, K., Lopaschuk, G. D., and Schulz, R. (2004) 'Pyruvate Prevents Cardiac Dysfunction and AMP-Activated Protein Kinase Activation by Hydrogen Peroxide in Isolated Rat Hearts'. *Canadian Journal of Physiology and Pharmacology* 82 (6), 409-416.

Levine, Y. C., Li, G. K., and Michel, T. (2007) 'Agonist-Modulated Regulation of AMP-Activated Protein Kinase (AMPK) in Endothelial Cells. Evidence for an AMPK -> Rac1 -> Akt -> Endothelial Nitric-Oxide Synthase Pathway'. *The Journal of Biological Chemistry* 282 (28), 20351-20364.

Lexis, C. P., van der Horst, Iwan CC, Lipsic, E., Wieringa, W. G., de Boer, R. A., van den Heuvel, Ad FM, van der Werf, Hindrik W, Schurer, R. A., Pundziute, G., and Tan, E. S. (2014) 'Effect of Metformin on Left Ventricular Function After Acute Myocardial Infarction in Patients without Diabetes: The GIPS-III Randomized Clinical Trial'. *Jama* 311 (15), 1526-1535.

Li, J., Brown, L. F., Hibberd, M. G., Grossman, J. D., Morgan, J. P., and Simons, M. (1996) 'VEGF, Flk-1, and Flt-1 Expression in a Rat Myocardial Infarction Model of Angiogenesis'. *The American Journal of Physiology* 270 (5 Pt 2), H1803-11.

Li, H., Yin, R., Chen, D., Liu, D., Wang, D., Yang, Q., and Dong, Y. (2007) 'Long-term Activation of Adenosine monophosphate-activated Protein Kinase Attenuates pressure-overload-induced Cardiac Hypertrophy'. *Journal of Cellular Biochemistry* 100 (5), 1086-1099.

Liang, H. and Ward, W. F. (2006) 'PGC-1alpha: A Key Regulator of Energy Metabolism'. *Advances in Physiology Education* 30 (4), 145-151.

Liedtke, A. J., DeMaison, L., Eggleston, A. M., Cohen, L. M., and Nellis, S. H. (1988) 'Changes in Substrate Metabolism and Effects of Excess Fatty Acids in Reperfused Myocardium'. *Circulation Research* 62 (3), 535-542.

Lin, H. V. and Accili, D. (2011) 'Hormonal Regulation of Hepatic Glucose Production in Health and Disease'. *Cell Metabolism* 14 (1), 9-19.

Linden, J. (2005) 'Adenosine in Tissue Protection and Tissue Regeneration'. *Molecular Pharmacology* 67 (5), 1385-1387.

Lipscombe, L. L., Goodwin, P. J., Zinman, B., McLaughlin, J. R., and Hux, J. E. (2008) 'The Impact of Diabetes on Survival Following Breast Cancer'. *Breast Cancer Research and Treatment* 109 (2), 389-395.

Liu, L., Cash, T. P., Jones, R. G., Keith, B., Thompson, C. B., and Simon, M. C. (2006) 'Hypoxia-Induced Energy Stress Regulates mRNA Translation and Cell Growth'. *Molecular Cell* 21 (4), 521-531.

Liu, B., Fan, Z., Edgerton, S. M., Deng, X., Alimova, I. N., Lind, S. E., and Thor, A. D. (2009) 'Metformin Induces Unique Biological and Molecular Responses in Triple Negative Breast Cancer Cells'. *Cell Cycle* 8 (13), 2031-2040.

Liu, J., Li, D., Zhou, J., Sun, L., Zhao, M., Kong, S., Wang, Y., Yu, X., Zhou, J., and Zang, W. (2011) 'Acetylcholine Prevents Angiotensin II-Induced Oxidative Stress and Apoptosis in H9c2 Cells'. *Apoptosis* 16 (1), 94-103.

Liu, X., Chhipa, R. R., Nakano, I., and Dasgupta, B. (2014) 'The AMPK Inhibitor Compound C is a Potent AMPK-Independent Antiglioma Agent'. *Molecular Cancer Therapeutics* 13 (3), 596-605.

Liu, C., Zhao, W., Meng, W., Zhao, T., Chen, Y., Ahokas, R. A., Liu, H., and Sun, Y. (2014) 'Platelet-Derived Growth Factor Blockade on Cardiac Remodeling Following Infarction'. *Molecular and Cellular Biochemistry* 397 (1-2), 295-304.

Liu, F., Zhang, W., Yang, F., Feng, T., Zhou, M., Yu, Y., Yu, X., Zhao, W., Yi, F., and Tang, W. (2016) 'Interleukin-6-Stimulated Progranulin Expression Contributes to the Malignancy of Hepatocellular Carcinoma Cells by Activating mTOR Signaling'. *Scientific Reports* 6, 21260.

Lizcano, J. M., Goransson, O., Toth, R., Deak, M., Morrice, N. A., Boudeau, J., Hawley, S. A., Udd, L., Makela, T. P., Hardie, D. G., and Alessi, D. R. (2004) 'LKB1 is a Master Kinase that Activates 13 Kinases of the AMPK Subfamily, Including MARK/PAR-1'. *The EMBO Journal* 23 (4), 833-843.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005) 'Rheb Binds and Regulates the mTOR Kinase'. *Current Biology* 15 (8), 702-713.

Longnus, S., Segalen, C., Giudicelli, J., Sajan, M., Farese, R., and Van Obberghen, E. (2005) 'Insulin Signalling Downstream of Protein Kinase B is Potentiated by 5′ AMP-Activated Protein Kinase in Rat Hearts in Vivo'. *Diabetologia* 48 (12), 2591-2601.

Lopaschuk, G. D., Spafford, M. A., Davies, N. J., and Wall, S. R. (1990) 'Glucose and Palmitate Oxidation in Isolated Working Rat Hearts Reperfused After a Period of Transient Global Ischemia'. *Circulation Research* 66 (2), 546-553.

Lopaschuk, G. D. and Saddik, M. (1992) 'The Relative Contribution of Glucose and Fatty Acids to ATP Production in Hearts Reperfused Following Ischemia'. in *Lipid Metabolism in the Healthy and Disease Heart*. ed. by Anon: Springer, 111-116.

Lu, H., Chen, C., and Klaassen, C. (2004) 'Tissue Distribution of Concentrative and Equilibrative Nucleoside Transporters in Male and Female Rats and Mice'. *Drug Metabolism and Disposition: The Biological Fate of Chemicals* 32 (12), 1455-1461.

Luft, D., Schmülling, R., and Eggstein, M. (1978) 'Lactic Acidosis in Biguanide-Treated Diabetics'. *Diabetologia* 14 (2), 75-87.

Lv, Q., Zhen, Q., Liu, L., Gao, R., Yang, S., Zhou, H., Goswami, R., and Li, Q. (2015) 'AMP-Kinase Pathway is Involved in Tumor Necrosis Factor Alpha-Induced Lipid Accumulation in Human Hepatoma Cells'. *Life Sciences* 131, 23-29.

MacDonald, M. R., Petrie, M. C., Varyani, F., Ostergren, J., Michelson, E. L., Young, J. B., Solomon, S. D., Granger, C. B., Swedberg, K., Yusuf, S., Pfeffer, M. A., McMurray, J. J., and CHARM Investigators (2008) 'Impact of Diabetes on Outcomes in Patients with Low and Preserved Ejection Fraction Heart Failure: An Analysis of the Candesartan in Heart Failure: Assessment of Reduction in Mortality and Morbidity (CHARM) Programme'. *European Heart Journal* 29 (11), 1377-1385.

Macmillan (2017), 'Statistics Fact Sheet'. *Macmillan cancer support*, [online], available from <https://www.macmillan.org.uk/_images/cancer-statistics-factsheet_tcm9-260514.pdf>, [26/April/2018].

Maddams, J., Utley, M., and Møller, H. (2012) 'Projections of Cancer Prevalence in the United Kingdom, 2010–2040'. *British Journal of Cancer* 107 (7), 1195.

Maddock, H. L., Mocanu, M. M., and Yellon, D. M. (2002) 'Adenosine A(3) Receptor Activation Protects the Myocardium from reperfusion/reoxygenation Injury'. *American Journal of Physiology.Heart and Circulatory Physiology* 283 (4), H1307-13.

Mahoney, S. J., Narayan, S., Molz, L., Berstler, L. A., Kang, S. A., Vlasuk, G. P., and Saiah, E. (2018) 'A Small Molecule Inhibitor of Rheb Selectively Targets mTORC1 Signaling'. *Nature Communications* 9 (1), 548.

Maida, A., Lamont, B., Cao, X., and Drucker, D. (2011) 'Metformin Regulates the Incretin Receptor Axis via a Pathway Dependent on Peroxisome Proliferator-Activated Receptor-α in Mice'. *Diabetologia* 54 (2), 339-349.

Malmberg, K., Yusuf, S., Gerstein, H. C., Brown, J., Zhao, F., Hunt, D., Piegas, L., Calvin, J., Keltai, M., and Budaj, A. (2000) 'Impact of Diabetes on Long-Term Prognosis in Patients with Unstable Angina and Non-Q-Wave Myocardial Infarction: Results of the OASIS (Organization to Assess Strategies for Ischemic Syndromes) Registry'. *Circulation* 102 (9), 1014-1019.

Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C., and Kroemer, G. (1998) 'Bax and Adenine Nucleotide Translocator Cooperate in the Mitochondrial Control of Apoptosis'. *Science (New York, N.Y.)* 281 (5385), 2027-2031.

Maas, A. and Appelman, Y. (2010) 'Gender Differences in Coronary Heart Disease'. *Netherlands Heart Journal* 18 (12), 598-603.

Maharsy, W., Aries, A., Mansour, O., Komati, H., and Nemer, M. (2014) 'Ageing is a Risk Factor in Imatinib Mesylate Cardiotoxicity'. *European Journal of Heart Failure* 16 (4), 367-376.

Matherne, G. P., Linden, J., Byford, A. M., Gauthier, N. S., and Headrick, J. P. (1997) 'Transgenic A1 Adenosine Receptor Overexpression Increases Myocardial

Resistance to Ischemia'. *Proceedings of the National Academy of Sciences of the United States of America* 94 (12), 6541-6546.

Matthews, D., Hosker, J., Rudenski, A., Naylor, B., Treacher, D., and Turner, R. (1985) 'Homeostasis Model Assessment: Insulin Resistance and β-Cell Function from Fasting Plasma Glucose and Insulin Concentrations in Man'. *Diabetologia* 28 (7), 412-419.

Maynard, S. E., Min, J. Y., Merchan, J., Lim, K. H., Li, J., Mondal, S., Libermann, T. A., Morgan, J. P., Sellke, F. W., Stillman, I. E., Epstein, F. H., Sukhatme, V. P., and Karumanchi, S. A. (2003) 'Excess Placental Soluble Fms-Like Tyrosine Kinase 1 (sFlt1) may Contribute to Endothelial Dysfunction, Hypertension, and Proteinuria in Preeclampsia'. *The Journal of Clinical Investigation* 111 (5), 649-658.

McBride, D., Alhazmi, A., Laovitthayanggoon, S., Boyd, M., and Currie, S. (2018). *6 Parallel Assessment of Cell Viability in Cardiac and Cancer Cells Following Treatment with Sunitinib.*

McGarry, J. D., Leatherman, G. F., and Foster, D. W. (1978) 'Carnitine Palmitoyltransferase I. the Site of Inhibition of Hepatic Fatty Acid Oxidation by Malonyl-CoA'. *The Journal of Biological Chemistry* 253 (12), 4128-4136.

McGuire, D., Emanuelsson, H., Granger, C., Ohman, E. M., Moliterno, D., White, H., Ardissino, D., Box, J., Califf, R., and Topol, E. (2000) 'Influence of Diabetes Mellitus on Clinical Outcomes Across the Spectrum of Acute Coronary Syndromes'. *Eur Heart J* 21 (21), 1750-1758.

McLeod, L. E. and Proud, C. G. (2002) 'ATP Depletion Increases Phosphorylation of Elongation Factor eEF2 in Adult Cardiomyocytes Independently of Inhibition of mTOR Signalling'. *FEBS Letters* 531 (3), 448-452.

Meissner, K., Heydrich, B., Jedlitschky, G., Schwabedissen, H. M. z., Mosyagin, I., Dazert, P., Eckel, L., Vogelgesang, S., Warzok, R. W., and Böhm, M. (2006) 'The ATP-Binding Cassette Transporter ABCG2 (BCRP), a Marker for Side Population Stem Cells, is Expressed in Human Heart'. *Journal of Histochemistry & Cytochemistry* 54 (2), 215-221.

Melnik, B. and Schmitz, G. (2014) 'Metformin: An Inhibitor of mTORC1 Signaling'. *J Endocrinol Diabetes Obes* 2 (2), 1029.

Mellor, H. R., Bell, A. R., Valentin, J., and Roberts, R. R. (2010) 'Cardiotoxicity Associated with Targeting Kinase Pathways in Cancer'. *Toxicological Sciences* 120 (1), 14-32.

Memmott, R. M. and Dennis, P. A. (2009) 'LKB1 and Mammalian Target of Rapamycin as Predictive Factors for the Anticancer Efficacy of Metformin'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 27 (34), e226; author reply e227.

Mena, A. C., Pulido, E. G., and Guillen-Ponce, C. (2010) 'Understanding the Molecular-Based Mechanism of Action of the Tyrosine Kinase Inhibitor: Sunitinib'. *Anti-Cancer Drugs* 21, S3-S11.

Mendel, D. B., Laird, A. D., Xin, X., Louie, S. G., Christensen, J. G., Li, G., Schreck, R. E., Abrams, T. J., Ngai, T. J., Lee, L. B., Murray, L. J., Carver, J., Chan, E., Moss, K. G., Haznedar, J. O., Sukbuntherng, J., Blake, R. A., Sun, L., Tang, C., Miller, T., Shirazian, S., McMahon, G., and Cherrington, J. M. (2003) 'In Vivo Antitumor Activity of SU11248, a Novel Tyrosine Kinase Inhibitor Targeting Vascular Endothelial Growth Factor and Platelet-Derived Growth Factor Receptors: Determination of a pharmacokinetic/pharmacodynamic Relationship'. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*9 (1), 327-337.

Menendez, J. A., Cufí, S., Oliveras-Ferraros, C., Martin-Castillo, B., Joven, J., Vellon, L., and Vazquez-Martin, A. (2011) 'Metformin and the ATM DNA Damage Response (DDR): Accelerating the Onset of Stress-Induced Senescence to Boost Protection against Cancer'. *Aging (Albany NY)* 3 (11), 1063-1077.

Mensah, K., Mocanu, M. M., and Yellon, D. M. (2005) 'Failure to Protect the Myocardium Acute Atorvastatin Treatment'. *Journal of the American College of Cardiology* 45 (8), 1287-1291.

Mercy, L., Pauw, A. d., Payen, L., Tejerina, S., Houbion, A., Demazy, C., Raes, M., Renard, P., and Arnould, T. (2005) 'Mitochondrial Biogenesis in mtDNA-depleted Cells Involves a Ca2 -dependent Pathway and a Reduced Mitochondrial Protein Import'. *The FEBS Journal* 272 (19), 5031-5055.

Mieulet, V. and Lamb, R. F. (2010) 'Tuberous Sclerosis Complex: Linking Cancer to Metabolism'. *Trends in Molecular Medicine* 16 (7), 329-335.

Miller, R. A., Chu, Q., Xie, J., Foretz, M., Viollet, B., and Birnbaum, M. J. (2013) 'Biguanides Suppress Hepatic Glucagon Signalling by Decreasing Production of Cyclic AMP'. *Nature* 494 (7436), 256-260.

Milojkovic, D. and Apperley, J. (2009) 'Mechanisms of Resistance to Imatinib and Second-Generation Tyrosine Inhibitors in Chronic Myeloid Leukemia'. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 15 (24), 7519-7527.

Ming, L., Wang, P., Bank, A., Yu, J., and Zhang, L. (2006) 'PUMA Dissociates Bax and Bcl-X(L) to Induce Apoptosis in Colon Cancer Cells'. *The Journal of Biological Chemistry* 281 (23), 16034-16042.

Minor, T. R., Rowe, M., Cullen, P. K., and Furst, S. (2008) 'Enhancing Brain Adenosine Signaling with the Nucleoside Transport Blocker NBTI (S-(4-Nitrobenzyl)-6-Theoinosine) Mimics the Effects of Inescapable Shock on Later Shuttle-Escape Performance in Rats.'. *Behavioral Neuroscience* 122 (6), 1236.

Misbin R.I., (2004). 'The phantom of lactic acidosis due to metformin in patients with diabetes. *Diabetes Care*, 27(7): pp. 1791-1793.

Mishra, P., Singh, S. V., Verma, A. K., Srivastava, P., Sultana, S., and Rath, S. K. (2014) 'Rosiglitazone Induces Cardiotoxicity by Accelerated Apoptosis'. *Cardiovascular Toxicology* 14 (2), 99-119.

Misirkic, M., Janjetovic, K., Vucicevic, L., Tovilovic, G., Ristic, B., Vilimanovich, U., Harhaji-Trajkovic, L., Sumarac-Dumanovic, M., Micic, D., and Bumbasirevic, V. (2012) 'Inhibition of AMPK-Dependent Autophagy Enhances in Vitro Antiglioma Effect of Simvastatin'. *Pharmacological Research* 65 (1), 111-119.

Mitchell, S., Ota, A., Foster, W., Zhang, B., Fang, Z., Patel, S., Nelson, S. F., Horvath, S., and Wang, Y. (2006) 'Distinct Gene Expression Profiles in Adult Mouse Heart Following Targeted MAP Kinase Activation'*. Physiological Genomics*25 (1), 50-59.

Mocanu, M. and Yellon, D. (2007) 'PTEN, the Achilles' Heel of Myocardial ischaemia/reperfusion Injury?'*. British Journal of Pharmacology* 150 (7), 833-838.

Monami, M., Lamanna, C., Balzi, D., Marchionni, N., and Mannucci, E. (2009) 'Sulphonylureas and Cancer: A case–control Study'*. Acta Diabetologica* 46 (4), 279.

Moore, F. and Brophy, P. J. (1994) 'Regulation of Acetyl-CoA Carboxylase (ACC) by ATP Depletion in Developing Oligodendrocytes Mimics the Action of AMP-Activated Protein Kinase (AMPK)'*. Biochemical Society Transactions* 22 (4), 416S.

Morabito, A., De Maio, E., Di Maio, M., Normanno, N., and Perrone, F. (2006) 'Tyrosine Kinase Inhibitors of Vascular Endothelial Growth Factor Receptors in Clinical Trials: Current Status and Future Directions'*. The Oncologist* 11 (7), 753-764.

Morin-Papunen, L., Rautio, K., Ruokonen, A., Hedberg, P., Puukka, M., and Tapanainen, J. S. (2003) 'Metformin Reduces Serum C-Reactive Protein Levels in Women with Polycystic Ovary Syndrome'*. The Journal of Clinical Endocrinology & Metabolism* 88 (10), 4649-4654.

Moslehi, J., Minamishima, Y. A., Shi, J., Neuberg, D., Charytan, D. M., Padera, R. F., Signoretti, S., Liao, R., and Kaelin, W. G.,Jr (2010) 'Loss of Hypoxia-Inducible Factor Prolyl Hydroxylase Activity in Cardiomyocytes Phenocopies Ischemic Cardiomyopathy'*. Circulation* 122 (10), 1004-1016.

Motzer, R. J., Michaelson, M. D., Redman, B. G., Hudes, G. R., Wilding, G., Figlin, R. A., Ginsberg, M. S., Kim, S. T., Baum, C. M., DePrimo, S. E., Li, J. Z., Bello, C. L., Theuer, C. P., George, D. J., and Rini, B. I. (2006) 'Activity of SU11248, a Multitargeted Inhibitor of Vascular Endothelial Growth Factor Receptor and Platelet-Derived Growth Factor Receptor, in Patients with Metastatic Renal Cell Carcinoma'.

Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology 24 (1), 16-24.

Motzer, R. J., Hutson, T. E., Tomczak, P., Michaelson, M. D., Bukowski, R. M., Rixe, O., Oudard, S., Negrier, S., Szczylik, C., and Kim, S. T. (2007) 'Sunitinib Versus Interferon Alfa in Metastatic Renal-Cell Carcinoma'*. New England Journal of Medicine* 356 (2), 115-124.

Motzer, R. J., Michaelson, M. D., Rosenberg, J., Bukowski, R. M., Curti, B. D., George, D. J., Hudes, G. R., Redman, B. G., Margolin, K. A., and Wilding, G. (2007) 'Sunitinib Efficacy Against Advanced Renal Cell Carcinoma'*. The Journal of Urology* 178 (5), 1883-1887.

Motzer RJ, Hutson TE, Cella D, Reeves J, Hawkins R, Guo J, Nathan P, Staehler M,
de Souza P, Merchan JR, Boleti E, Fife K, Jin J, Jones R, Uemura H, De Giorgi U, Harmenberg U, Wang J, Sternberg CN, Deen K, McCann L, Hackshaw MD,
Crescenzo R, Pandite LN, Choueiri TK. (2013) 'Pazopanib versus sunitinib in metastatic
Renal-cell carcinoma. N Engl J Med; 369: 722 – 731.

Mu, J., Brozinick, J. T., Valladares, O., Bucan, M., and Birnbaum, M. J. (2001) 'A Role for AMP-Activated Protein Kinase in Contraction-and Hypoxia-Regulated Glucose Transport in Skeletal Muscle'*. Molecular Cell* 7 (5), 1085-1094.

Mubagwa, K., Mullane, K., and Flameng, W. (1996) 'Role of Adenosine in the Heart and Circulation'*. Cardiovascular Research* 32 (5), 797-813.

Mubagwa, K. and Flameng, W. (2001) 'Adenosine, Adenosine Receptors and Myocardial Protection: An Updated Overview'*. Cardiovascular Research* 52 (1), 25-39.

Muders, M. H., Zhang, H., Wang, E., Tindall, D. J., and Datta, K. (2009) 'Vascular Endothelial Growth Factor-C Protects Prostate Cancer Cells from Oxidative Stress by the Activation of Mammalian Target of Rapamycin Complex-2 and AKT-1'*. Cancer Research* 69 (15), 6042-6048.

Murphy, E. and Steenbergen, C. (2008) 'Mechanisms Underlying Acute Protection from Cardiac Ischemia-Reperfusion Injury'. *Physiological Reviews* 88 (2), 581-609.

Musi, N., Hirshman, M. F., Arad, M., Xing, Y., Fujii, N., Pomerleau, J., Ahmad, F., Berul, C. I., Seidman, J. G., and Tian, R. (2005) 'Functional Role of AMP-activated Protein Kinase in the Heart during Exercise'. *FEBS Letters* 579 (10), 2045-2050.

Nadtochiy, S. M., Burwell, L. S., and Brookes, P. S. (2007) 'Cardioprotection and Mitochondrial S-Nitrosation: Effects of S-Nitroso-2-Mercaptopropionyl Glycine (SNO-MPG) in Cardiac ischemia–reperfusion Injury'. *Journal of Molecular and Cellular Cardiology* 42 (4), 812-825.

Nair, S., Diehl, A., Wiseman, M., Farr, G., and Perrillo, R. (2004) 'Metformin in the Treatment of non-alcoholic Steatohepatitis: A Pilot Open Label Trial'. *Alimentary Pharmacology & Therapeutics* 20 (1), 23-28.

Najjar, S. M., Yang, Y., Fernström, M. A., Lee, S., DeAngelis, A. M., Rjaily, G. A. A., Al-Share, Q. Y., Dai, T., Miller, T. A., and Ratnam, S. (2005) 'Insulin Acutely Decreases Hepatic Fatty Acid Synthase Activity'. *Cell Metabolism* 2 (1), 43-53.

Narayan, V., Keefe, S., Haas, N., Wang, L., Puzanov, I., Putt, M., Catino, A., Fang, J., Agarwal, N., Hyman, D., Smith, A. M., Finkelman, B. S., Narayan, H. K., Ewer, S., ElAmm, C., Lenihan, D., and Ky, B. (2017) 'Prospective Evaluation of Sunitinib-Induced Cardiotoxicity in Patients with Metastatic Renal Cell Carcinoma'. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 23 (14), 3601-3609.

Nath, N., Khan, M., Paintlia, M. K., Singh, I., Hoda, M. N., and Giri, S. (2009) 'Metformin Attenuated the Autoimmune Disease of the Central Nervous System in Animal Models of Multiple Sclerosis'. *Journal of Immunology (Baltimore, Md.: 1950)* 182 (12), 8005-8014.

National Heart, Lung and Blood Institute (2014) 'High Blood Cholesterol". *National Heart, Lung and Blood Institute*. [online] available from < https://www.nhlbi.nih.gov/health-topics/high-blood-cholesterol> [26th June 2018].

National Heart, Lung and Blood Institute (2017) ‘What Are the Risk Factors for Heart Disease?’, *National Heart, Lung and Blood Institute* [online] available from <https://www.nhlbi.nih.gov/health/educational/hearttruth/lower-risk/risk-factors.htm> [26th June 2018].

National Heart, Lung and Blood Institute (2018) ‘High Blood Pressure”. *National Heart, Lung and Blood Institute*. [online] available from <https://www.nhlbi.nih.gov/health-topics/high-blood-pressure> [26th June 2018].

National Institute for Health and Care Excellence (2019) ‘METFORMIN HYDROCHLORIDE’, *National Institute for Health and Care Excellence*. [online] available from < https://bnf.nice.org.uk/drug/metformin-hydrochloride.html> [1st June 2019].

Neely, J. R. and Morgan, H. E. (1974) 'Relationship between Carbohydrate and Lipid Metabolism and the Energy Balance of Heart Muscle'*. Annual Review of Physiology* 36 (1), 413-459.

Nelson, J. F., Strong, R., Bokov, A., Diaz, V., and Ward, W. (2012) 'Probing the Relationship between Insulin Sensitivity and Longevity using Genetically Modified Mice'*. The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences*67 (12), 1332-1338.

Neshat, M. S., Mellinghoff, I. K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J. J., Wu, H., and Sawyers, C. L. (2001) 'Enhanced Sensitivity of PTEN-Deficient Tumors to Inhibition of FRAP/mTOR'*. Proceedings of the National Academy of Sciences of the United States of America* 98 (18), 10314-10319.

Nies, A. T., Damme, K., Schaeffeler, E., and Schwab, M. (2012) 'Multidrug and Toxin Extrusion Proteins as Transporters of Antimicrobial Drugs'*. Expert Opinion on Drug Metabolism & Toxicology* 8 (12), 1565-1577.

Ning, J. and Clemmons, D. R. (2010) 'AMP-Activated Protein Kinase Inhibits IGF-I Signaling and Protein Synthesis in Vascular Smooth Muscle Cells Via Stimulation of Insulin Receptor Substrate 1 S794 and Tuberous Sclerosis 2 S1345 Phosphorylation'*. Molecular Endocrinology* 24 (6), 1218-1229.

Oeffinger, K. C., Mertens, A. C., Sklar, C. A., Kawashima, T., Hudson, M. M., Meadows, A. T., Friedman, D. L., Marina, N., Hobbie, W., and Kadan-Lottick, N. S. (2006) 'Chronic Health Conditions in Adult Survivors of Childhood Cancer'. *New England Journal of Medicine* 355 (15), 1572-1582.

Oidor-Chan, V. H., Hong, E., Pérez-Severiano, F., Montes, S., Torres-Narváez, J. C., del Valle-Mondragón, L., Pastelín-Hernández, G., and Sánchez-Mendoza, A. (2016) 'Fenofibrate Plus Metformin Produces Cardioprotection in a Type 2 Diabetes and Acute Myocardial Infarction Model'. *PPAR Research* 2016.

O'Farrell, A. M., Abrams, T. J., Yuen, H. A., Ngai, T. J., Louie, S. G., Yee, K. W., Wong, L. M., Hong, W., Lee, L. B., Town, A., Smolich, B. D., Manning, W. C., Murray, L. J., Heinrich, M. C., and Cherrington, J. M. (2003) 'SU11248 is a Novel FLT3 Tyrosine Kinase Inhibitor with Potent Activity in Vitro and in Vivo'. *Blood* 101 (9), 3597-3605.

Olsson, J., Lindberg, G., Gottsäter, M., Lindwall, K., Sjöstrand, Å., Tisell, A., and Melander, A. (2000) 'Increased Mortality in Type II Diabetic Patients using Sulphonylurea and Metformin in Combination: A Population-Based Observational Study'. *Diabetologia* 43 (5), 558-560.

Orphanos, G. S., Ioannidis, G. N., and Ardavanis, A. G. (2009) 'Cardiotoxicity Induced by Tyrosine Kinase Inhibitors'. *Acta Oncologica* 48 (7), 964-970.

Owen, M., Doran, E., and Halestrap, A. (2000) 'Evidence that Metformin Exerts its Anti-Diabetic Effects through Inhibition of Complex 1 of the Mitochondrial Respiratory Chain'. *Biochem.J* 348, 607-614.

Oxford University Hospitals (2014)., “Von Hippel-lindau Disease, An information leaflet for patietns and families”. *Oxford University Hospitals NHS Trust Oxford Regional Genetic Department* [online] available from <https://www.ouh.nhs.uk/patient-guide/leaflets/files/10059Pvonhippellindau.pdf> [12th July 2018].

Ozawa, K., Kondo, T., Hori, O., Kitao, Y., Stern, D. M., Eisenmenger, W., Ogawa, S., and Ohshima, T. (2001) 'Expression of the Oxygen-Regulated Protein ORP150

Accelerates Wound Healing by Modulating Intracellular VEGF Transport'. *The Journal of Clinical Investigation* 108 (1), 41-50.

Pastor-Anglada, M., Molina-Arcas, M., Casado, F., Bellosillo, B., Colomer, D., and Gil, J. (2004) 'Nucleoside Transporters in Chronic Lymphocytic Leukaemia'. *Leukemia* 18 (3), 385-393.

Paiva, M., Riksen, N. P., Davidson, S. M., Hausenloy, D. J., Monteiro, P., Goncalves, L., Providencia, L., Rongen, G. A., Smits, P., Mocanu, M. M., and Yellon, D. M. (2009) 'Metformin Prevents Myocardial Reperfusion Injury by Activating the Adenosine Receptor'. *Journal of Cardiovascular Pharmacology* 53 (5), 373-378.

Paiva, M. A., Gonçalves, L. M., Providência, L. A., Davidson, S. M., Yellon, D. M., and Mocanu, M. M. (2010) 'Transitory Activation of AMPK at Reperfusion Protects the Ischaemic-Reperfused Rat Myocardium Against Infarction'. *Cardiovascular Drugs and Therapy* 24 (1), 25-32.

Page, R. L., O'Bryant, C. L., Cheng, D., Dow, T. J., Ky, B., Stein, C. M., Spencer, A. P., Trupp, R. J., and Lindenfeld, J. (2016) 'Drugs that may Cause Or Exacerbate Heart Failure: A Scientific Statement from the American Heart Association'. *Circulation* 134 (6), e32-e69.

Pan, D., Dong, J., Zhang, Y., and Gao, X. (2004) 'Tuberous Sclerosis Complex: From Drosophila to Human Disease'. *Trends in Cell Biology* 14 (2), 78-85.

Patel, P. H., Senico, P. L., Curiel, R. E., and Motzer, R. J. (2009) 'Phase I Study Combining Treatment with Temsirolimus and Sunitinib Malate in Patients with Advanced Renal Cell Carcinoma'. *Clinical Genitourinary Cancer* 7 (1), 24-27.

Patel, S., Singh, N., and Kumar, L. (2015) 'Evaluation of Effects of Metformin in Primary Ovarian Cancer Cells'. *Asian Pacific Journal of Cancer Prevention : APJCP* 16 (16), 6973-6979.

Pathania, A. S., Guru, S. K., Verma, M., Sharma, C., Abdullah, S. T., Malik, F., Chandra, S., Katoch, M., and Bhushan, S. (2013) 'Disruption of the PI3K/AKT/mTOR Signaling Cascade and Induction of Apoptosis in HL-60 Cells by

an Essential Oil from Monarda Citriodora'. *Food and Chemical Toxicology* 62, 246-254.

Paul, B. Y., Hong, C. C., Sachidanandan, C., Babitt, J. L., Deng, D. Y., Hoyng, S. A., Lin, H. Y., Bloch, K. D., and Peterson, R. T. (2008) 'Dorsomorphin Inhibits BMP Signals Required for Embryogenesis and Iron Metabolism'. *Nature Chemical Biology* 4 (1), 33-41.

Pauriah, M., Struthers, A. D., and Lang, C. C. (2008) 'Biomarkers and Surrogate Endpoints in Cardiovascular Therapeutics Research: Under Scrutiny Following Results of the ENHANCE Study'. *Cardiovascular Therapeutics* 26 (2), 85-88.

Peart, J. N. and Headrick, J. P. (2007) 'Adenosinergic Cardioprotection: Multiple Receptors, Multiple Pathways'. *Pharmacology & Therapeutics* 114 (2), 208-221.

Pelleg, A., Pennock, R. S., and Kutalek, S. P. (2002) 'Proarrhythmic Effects of Adenosine: One Decade of Clinical Data'. *American Journal of Therapeutics* 9 (2), 141-147.

Pennycooke, M., Chaudary, N., Shuralyova, I., Zhang, Y., and Coe, I. R. (2001) 'Differential Expression of Human Nucleoside Transporters in Normal and Tumor Tissue'. *Biochemical and Biophysical Research Communications*280 (3), 951-959.

Pernicova, I. and Korbonits, M. (2014) 'Metformin - Mode of Action and Clinical Implications for Diabetes and Cancer'. *Nature Reviews Endocrinology* 10 (3), 143-156.

Pfizer (2014) 'Safety Data Sheet Sunitinib Malate Capsules'. *Pfizer*. Version 3.0. [online] available from <http://www.pfizer.com/sites/default/files/products/material_safety_data/Sunitinib_Capsules_27-MAR-2014.pdf> [29th December 2015].

Piccart-Gebhart, M. J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I., Gianni, L., Baselga, J., Bell, R., and Jackisch, C. (2005) 'Trastuzumab After Adjuvant Chemotherapy in HER2-Positive Breast Cancer'. *New England Journal of Medicine* 353 (16), 1659-1672.

Pierotti, M., Berrino, F., Gariboldi, M., Melani, C., Mogavero, A., Negri, T., Pasanisi, P., and Pilotti, S. (2013) 'Targeting Metabolism for Cancer Treatment and Prevention: Metformin, an Old Drug with Multi-Faceted Effects'. *Oncogene* 32 (12), 1475-1487.

Pikiou, O., Vasilaki, A., Leondaritis, G., Vamvakopoulos, N., and Messinis, I. E. (2015) 'Effects of Metformin on Fertilisation of Bovine Oocytes and Early Embryo Development: Possible Involvement of AMPK3-Mediated TSC2 Activation'. *Zygote* 23 (01), 58-67.

Piper, H. M., Garcia-Dorado, D., and Ovize, M. (1998) 'A Fresh Look at Reperfusion Injury'. *Cardiovascular Research* 38 (2), 291-300.

Polekhina, G., Gupta, A., Michell, B. J., van Denderen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., and Parker, M. W. (2003) 'AMPK β Subunit Targets Metabolic Stress Sensing to Glycogen'. *Current Biology* 13 (10), 867-871.

Pollak, M. N. (2012) 'Investigating Metformin for Cancer Prevention and Treatment: The End of the Beginning'. *Cancer Discovery* 2 (9), 778-790.

Porta, C. and Chiellino, S. (2016) 'ASSURE Vs. S-TRAC: Conflicting Results of Adjuvant Treatments for Kidney Cancer in the Era of Targeted Agents and Genomics'. *Annals of Translational Medicine* 4 (Suppl 1).

Potter, C. J., Huang, H., and Xu, T. (2001) 'Drosophila Tsc1 Functions with Tsc2 to Antagonize Insulin Signaling in Regulating Cell Growth, Cell Proliferation, and Organ Size'. *Cell* 105 (3), 357-368.

Powles, T., Chowdhury, S., Jones, R., Mantle, M., Nathan, P., Bex, A., Lim, L., and Hutson, T. (2011) 'Sunitinib and Other Targeted Therapies for Renal Cell Carcinoma'. *British Journal of Cancer* 104 (5), 741.

Preiss, D., Lloyd, S. M., Ford, I., McMurray, J. J., Holman, R. R., Welsh, P., Fisher, M., Packard, C. J., and Sattar, N. (2014) 'Metformin for Non-Diabetic Patients with Coronary Heart Disease (the CAMERA Study): A Randomised Controlled Trial'. *The Lancet Diabetes & Endocrinology* 2 (2), 116-124.

Qi, D. and Young, L. H. (2015) 'AMPK: Energy Sensor and Survival Mechanism in the Ischemic Heart'. *Trends in Endocrinology & Metabolism* 26 (8), 422-429.

Qi WX, Shen Z, Tang LN, Yao Y. Congestive heart failure risk in cancer patients treated with vascular endothelial growth factor tyrosine kinase inhibitors: a systematic review and meta-analysis of 36 clinical trials. Br J Clin Pharmacol 2014; 78:748–762.

Rabinowitz, J. D. and White, E. (2010) 'Autophagy and Metabolism'. *Science (New York, N.Y.)* 330 (6009), 1344-1348.

Rafaeloff-Phail, R., Ding, L., Conner, L., Yeh, W. K., McClure, D., Guo, H., Emerson, K., and Brooks, H. (2004) 'Biochemical Regulation of Mammalian AMP-Activated Protein Kinase Activity by NAD and NADH'. *The Journal of Biological Chemistry* 279 (51), 52934-52939.

Ramjane, K., Han, L., and Jin, C. (2008) 'The Diagnosis and Treatment of the no-Reflow Phenomenon in Patients with Myocardial Infarction Undergoing Percutaneous Coronary Intervention'. *Experimental and Clinical Cardiology* 13 (3), 121-128.

RCOG (2014) 'Long-term Consequences of Polycystic Ovary Sundrome; Green-top Guideline No. 33' *Royal College of Obstetricians & Gynaecologists*. [online] available from <https://www.rcog.org.uk/globalassets/documents/guidelines/gtg_33.pdf> [01/03/2015].

Reiling, J. H. and Hafen, E. (2004) 'The Hypoxia-Induced Paralogs Scylla and Charybdis Inhibit Growth by Down-Regulating S6K Activity Upstream of TSC in Drosophila'. *Genes & Development* 18 (23), 2879-2892.

Ren, J., Dominguez, L. J., Sowers, J. R., and Davidoff, A. J. (1999) 'Metformin but Not Glyburide Prevents High Glucose-Induced Abnormalities in Relaxation and Intracellular Ca2+ Transients in Adult Rat Ventricular Myocytes'. *Diabetes* 48 (10), 2059-2065.

Rena, G., Pearson, E. R., and Sakamoto, K. (2013) 'Molecular Mechanism of Action of Metformin: Old Or New Insights?'. *Diabetologia* 56 (9), 1898-1906.

Rios, M., Foretz, M., Viollet, B., Prieto, A., Fraga, M., Costoya, J. A., and Senaris, R. (2013) 'AMPK Activation by Oncogenesis is Required to Maintain Cancer Cell Proliferation in Astrocytic Tumors'. *Cancer Research* 73 (8), 2628-2638.

Roberts, D. J., Tan-Sah, V. P., Ding, E. Y., Smith, J. M., and Miyamoto, S. (2014) 'Hexokinase-II Positively Regulates Glucose Starvation-Induced Autophagy through TORC1 Inhibition'. *Molecular Cell* 53 (4), 521-533.

Rocha, G. Z., Dias, M. M., Ropelle, E. R., Osorio-Costa, F., Rossato, F. A., Vercesi, A. E., Saad, M. J., and Carvalheira, J. B. (2011) 'Metformin Amplifies Chemotherapy-Induced AMPK Activation and Antitumoral Growth'. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 17 (12), 3993-4005.

Rogalska, A., Sliwinska, A., Kasznicki, J., Drzewoski, J., and Marczak, A. (2016) 'Effects of Epothilone A in Combination with the Antidiabetic Drugs Metformin and Sitagliptin in HepG2 Human Hepatocellular Cancer Cells: Role of Transcriptional Factors NF-κB and p53'. *Asian Pacific Journal of Cancer Prevention* 17 (3), 993-1001.

Rosamond, W., Flegal, K., Friday, G., Furie, K., Go, A., Greenlund, K., Haase, N., Ho, M., Howard, V., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C. J., Roger, V., Rumsfeld, J., Sorlie, P., Steinberger, J., Thom, T., Wasserthiel-Smoller, S., Hong, Y., and American Heart Association Statistics Committee and Stroke Statistics Subcommittee (2007) 'Heart Disease and Stroke Statistics--2007 Update: A Report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee'. *Circulation* 115 (5), e69-171.

Rose, J. B., Naydenova, Z., Bang, A., Eguchi, M., Sweeney, G., Choi, D. S., Hammond, J. R., and Coe, I. R. (2010) 'Equilibrative Nucleoside Transporter 1 Plays an Essential Role in Cardioprotection'. *American Journal of Physiology.Heart and Circulatory Physiology* 298 (3), H771-7.

Rosen, P. and Wiernsperger, N. F. (2006) 'Metformin Delays the Manifestation of Diabetes and Vascular Dysfunction in Goto–Kakizaki Rats by Reduction of Mitochondrial Oxidative Stress'. *Diabetes/metabolism Research and Reviews* 22 (4), 323-330.

Roskoski Jr, R. (2016) 'Classification of Small Molecule Protein Kinase Inhibitors Based upon the Structures of their Drug-Enzyme Complexes'. *Pharmacological Research* 103, 26-48.

Ruiz-Meana, M., Garcia-Dorado, D., Miro-Casas, E., Abellan, A., and Soler-Soler, J. (2006) 'Mitochondrial Ca2+ Uptake during Simulated Ischemia does Not Affect Permeability Transition Pore Opening upon Simulated Reperfusion'. *Cardiovascular Research* 71 (4), 715-724.

Russell, R. R.,3rd, Li, J., Coven, D. L., Pypaert, M., Zechner, C., Palmeri, M., Giordano, F. J., Mu, J., Birnbaum, M. J., and Young, L. H. (2004) 'AMP-Activated Protein Kinase Mediates Ischemic Glucose Uptake and Prevents Postischemic Cardiac Dysfunction, Apoptosis, and Injury'. *The Journal of Clinical Investigation* 114 (4), 495-503.

Saeedi, R., Parsons, H. L., Wambolt, R. B., Paulson, K., Sharma, V., Dyck, J. R., Brownsey, R. W., and Allard, M. F. (2008) 'Metabolic Actions of Metformin in the Heart can Occur by AMPK-Independent Mechanisms'. *American Journal of Physiology.Heart and Circulatory Physiology* 294 (6), H2497-506.

Saddik, M. and Lopaschuk, G. D. (1991) 'Myocardial Triglyceride Turnover and Contribution to Energy Substrate Utilization in Isolated Working Rat Hearts'. *The Journal of Biological Chemistry* 266 (13), 8162-8170.

Saddik, M. and Lopaschuk, G. D. (1992) 'Myocardial Triglyceride Turnover during Reperfusion of Isolated Rat Hearts Subjected to a Transient Period of Global Ischemia'. *The Journal of Biological Chemistry* 267 (6), 3825-3831.

Sahin, F., Kannangai, R., Adegbola, O., Wang, J., Su, G., and Torbenson, M. (2004) 'MTOR and P70 S6 Kinase Expression in Primary Liver Neoplasms'. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 10 (24), 8421-8425.

Sai, K., Yang, D., Yamamoto, H., Fujikawa, H., Yamamoto, S., Nagata, T., Saito, M., Yamamura, T., and Nishizaki, T. (2006) 'A1 Adenosine Receptor Signal and AMPK Involving Caspase-9/-3 Activation are Responsible for Adenosine-Induced RCR-1 Astrocytoma Cell Death'. *Neurotoxicology* 27 (4), 458-467.

Saito, T., Chiba, T., Yuki, K., Zen, Y., Oshima, M., Koide, S., Motoyama, T., Ogasawara, S., Suzuki, E., and Ooka, Y. (2013) 'Metformin, a Diabetes Drug, Eliminates Tumor-Initiating Hepatocellular Carcinoma Cells'. *PloS One* 8 (7), e70010.

Sakamoto, K., Goransson, O., Hardie, D. G., and Alessi, D. R. (2004) 'Activity of LKB1 and AMPK-Related Kinases in Skeletal Muscle: Effects of Contraction, Phenformin, and AICAR'. *American Journal of Physiology.Endocrinology and Metabolism* 287 (2), E310-7.

Sakamoto, K., Zarrinpashneh, E., Budas, G. R., Pouleur, A., Dutta, A., Prescott, A. R., Vanoverschelde, J., Ashworth, A., Jovanovic, A., and Alessi, D. R. (2006) 'Deficiency of LKB1 in Heart Prevents Ischemia-Mediated Activation of AMPKα2 but Not AMPKα1'. *American Journal of Physiology-Endocrinology and Metabolism* 290 (5), E780-E788.

Salpeter, S. R., Buckley, N. S., Kahn, J. A., and Salpeter, E. E. (2008) 'Meta-Analysis: Metformin Treatment in Persons at Risk for Diabetes Mellitus'. *The American Journal of Medicine* 121 (2), 149-157. e2.

Sambol, N. C., Chiang, J., O'Conner, M., Liu, C. Y., Lin, E. T., Goodman, A. M., Benet, L. Z., and Karam, J. H. (1996) 'Pharmacokinetics and Pharmacodynamics of Metformin in Healthy Subjects and Patients with noninsulin-dependent Diabetes Mellitus'. *The Journal of Clinical Pharmacology* 36 (11), 1012-1021.

Sanada, S., Komuro, I., and Kitakaze, M. (2011) 'Pathophysiology of Myocardial Reperfusion Injury: Preconditioning, Postconditioning, and Translational Aspects of Protective Measures'. *American Journal of Physiology.Heart and Circulatory Physiology* 301 (5), H1723-41.

Sandhu, H., Cooper, S., Hussain, A., Mee, C., and Maddock, H. (2017) 'Attenuation of Sunitinib-Induced Cardiotoxicity through the A3 Adenosine Receptor Activation'. *European Journal of Pharmacology* 814, 95-105.

Sanli, T., Rashid, A., Liu, C., Harding, S., Bristow, R. G., Cutz, J., Singh, G., Wright, J., and Tsakiridis, T. (2010) 'Ionizing Radiation Activates AMP-Activated Kinase (AMPK): A Target for Radiosensitization of Human Cancer Cells'. *International Journal of Radiation Oncology* Biology* Physics* 78 (1), 221-229.

Sarbassov, D. D., Ali, S. M., and Sabatini, D. M. (2005) 'Growing Roles for the mTOR Pathway'. *Current Opinion in Cell Biology* 17 (6), 596-603.

Sardão, V. A., Oliveira, P. J., Holy, J., Oliveira, C. R., and Wallace, K. B. (2009) 'Morphological Alterations Induced by Doxorubicin on H9c2 Myoblasts: Nuclear, Mitochondrial, and Cytoskeletal Targets'. *Cell Biology and Toxicology* 25 (3), 227-243.

Sartoretto, J. L., Melo, G. A., Carvalho, M. H., Nigro, D., Passaglia, R. T., Scavone, C., Cuman, R. K., and Fortes, Z. B. (2005) 'Metformin Treatment Restores the Altered Microvascular Reactivity in Neonatal Streptozotocin-Induced Diabetic Rats Increasing NOS Activity, but Not NOS Expression'. *Life Sciences* 77 (21), 2676-2689.

Sasaki, H., Asanuma, H., Fujita, M., Takahama, H., Wakeno, M., Ito, S., Ogai, A., Asakura, M., Kim, J., Minamino, T., Takashima, S., Sanada, S., Sugimachi, M., Komamura, K., Mochizuki, N., and Kitakaze, M. (2009) 'Metformin Prevents Progression of Heart Failure in Dogs: Role of AMP-Activated Protein Kinase'. *Circulation* 119 (19), 2568-2577.

Schlessinger, J. (2000) 'Cell Signaling by Receptor Tyrosine Kinases'. *Cell* 103 (2), 211-225.

Schreiber, S. N., Knutti, D., Brogli, K., Uhlmann, T., and Kralli, A. (2003) 'The Transcriptional Coactivator PGC-1 Regulates the Expression and Activity of the Orphan Nuclear Receptor Estrogen-Related Receptor Alpha (ERRalpha)'. *The Journal of Biological Chemistry* 278 (11), 9013-9018.

Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G., and Hardie, D. G. (2004) 'CBS Domains Form Energy-Sensing Modules Whose Binding of Adenosine Ligands is Disrupted by Disease Mutations'. *The Journal of Clinical Investigation* 113 (2), 274-284.

Segal, E. D., Yasmeen, A., Beauchamp, M., Rosenblatt, J., Pollak, M., and Gotlieb, W. H. (2011) 'Relevance of the OCT1 Transporter to the Antineoplastic Effect of Biguanides'. *Biochemical and Biophysical Research Communications* 414 (4), 694-699.

Selvin, E., Bolen, S., Yeh, H., Wiley, C., Wilson, L. M., Marinopoulos, S. S., Feldman, L., Vassy, J., Wilson, R., and Bass, E. B. (2008) 'Cardiovascular Outcomes in Trials of Oral Diabetes Medications: A Systematic Review'. *Archives of Internal Medicine* 168 (19), 2070-2080.

Semenza, G. L. (2003) 'Targeting HIF-1 for Cancer Therapy'. *Nature Reviews Cancer* 3 (10), 721.

Sengupta, S., Peterson, T. R., and Sabatini, D. M. (2010) 'Regulation of the mTOR Complex 1 Pathway by Nutrients, Growth Factors, and Stress'. *Molecular Cell* 40 (2), 310-322.

Shackelford, D. B. and Shaw, R. J. (2009) 'The LKB1-AMPK Pathway: Metabolism and Growth Control in Tumour Suppression'. *Nature Reviews.Cancer* 9 (8), 563-575.

Shaker, M., Mashhadani, Z. I., and Mehdi, A. A. (2010) 'Effect of Treatment with Metformin on Omentin-1, Ghrelin and Other Biochemical, Clinical Features in PCOS Patients'. *Oman Medical Journal* 25 (4), 289-293.

Shang, L., Chen, S., Du, F., Li, S., Zhao, L., and Wang, X. (2011) 'Nutrient Starvation Elicits an Acute Autophagic Response Mediated by Ulk1 Dephosphorylation and its Subsequent Dissociation from AMPK'. *Proceedings of the National Academy of Sciences of the United States of America* 108 (12), 4788-4793.

Shaw, R. J., Bardeesy, N., Manning, B. D., Lopez, L., Kosmatka, M., DePinho, R. A., and Cantley, L. C. (2004) 'The LKB1 Tumor Suppressor Negatively Regulates mTOR Signaling'. *Cancer Cell* 6 (1), 91-99.

Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (2004) 'The Tumor Suppressor LKB1 Kinase Directly Activates AMP-Activated Kinase and Regulates Apoptosis in Response to Energy Stress'. *Proceedings of the National Academy of Sciences of the United States of America* 101 (10), 3329-3335.

Shaw, R. J., Lamia, K. A., Vasquez, D., Koo, S. H., Bardeesy, N., Depinho, R. A., Montminy, M., and Cantley, L. C. (2005) 'The Kinase LKB1 Mediates Glucose Homeostasis in Liver and Therapeutic Effects of Metformin'. *Science (New York, N.Y.)* 310 (5754), 1642-1646.

Shi, W., Xiao, D., Wang, L., Dong, L., Yan, Z., Shen, Z., Chen, S., Chen, Y., and Zhao, W. (2012) 'Therapeutic metformin/AMPK Activation Blocked Lymphoma Cell Growth Via Inhibition of mTOR Pathway and Induction of Autophagy'. *Cell Death & Disease* 3 (3), e275.

Shibata, R., Sato, K., Pimentel, D. R., Takemura, Y., Kihara, S., Ohashi, K., Funahashi, T., Ouchi, N., and Walsh, K. (2005) 'Adiponectin Protects Against Myocardial Ischemia-Reperfusion Injury through AMPK-and COX-2–dependent Mechanisms'. *Nature Medicine* 11 (10), 1096-1103.

Shimizu, T., Cao, C. X., Shao, R. G., and Pommier, Y. (1998) 'Lamin B Phosphorylation by Protein Kinase Calpha and Proteolysis during Apoptosis in Human Leukemia HL60 Cells'. *The Journal of Biological Chemistry* 273 (15), 8669-8674.

Shoelson, S. E., Lee, J., and Goldfine, A. B. (2006) 'Inflammation and Insulin Resistance'. *The Journal of Clinical Investigation* 116 (7), 1793-1801.

Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin.* 2016;66:7-30.

Sieghart, W., Fuereder, T., Schmid, K., Cejka, D., Werzowa, J., Wrba, F., Wang, X., Gruber, D., Rasoul-Rockenschaub, S., and Peck-Radosavljevic, M. (2007)

'Mammalian Target of Rapamycin Pathway Activity in Hepatocellular Carcinomas of Patients Undergoing Liver Transplantation'. *Transplantation* 83 (4), 425-432.

Siegmund, B., Ladilov, Y. V., and Piper, H. M. (1994) 'Importance of Sodium for Recovery of Calcium Control in Reoxygenated Cardiomyocytes'. *The American Journal of Physiology* 267 (2 Pt 2), H506-13.

Silke, B., Campbell, C., and King, D. (2002) 'The Potential Cardiotoxicity of Antipsychotic Drugs as Assessed by Heart Rate Variability'. *Journal of Psychopharmacology* 16 (4), 355-360.

Sliwinska, A., Rogalska, A., Marczak, A., Kasznicki, J., and Drzewoski, J. (2015) 'Metformin, but Not Sitagliptin, Enhances WP 631-Induced Apoptotic HepG2 Cell Death'. *Toxicology in Vitro* 29 (5), 1116-1123.

Silvestre, J., Carvalho, S., Mendes, V., Coelho, L., Tapadinhas, C., Ferreira, P., Povoa, P., and Ceia, F. (2007) 'Metformin-Induced Lactic Acidosis: A Case Series'. *Journal of Medical Case Reports* 1 (1), 126.

Skinner, H. D., Crane, C. H., Garrett, C. R., Eng, C., Chang, G. J., Skibber, J. M., Rodriguez-Bigas, M. A., Kelly, P., Sandulache, V. C., and Delclos, M. E. (2013) 'Metformin use and Improved Response to Therapy in Rectal Cancer'. *Cancer Medicine* 2 (1), 99-107.

Skrzypiec-Spring, M., Grotthus, B., Szeląg, A., and Schulz, R. (2007) 'Isolated Heart Perfusion According to Langendorff—still Viable in the New Millennium'. *Journal of Pharmacological and Toxicological Methods* 55 (2), 113-126.

Spector, N. L., Yarden, Y., Smith, B., Lyass, L., Trusk, P., Pry, K., Hill, J. E., Xia, W., Seger, R., and Bacus, S. S. (2007) 'Activation of AMP-Activated Protein Kinase by Human EGF Receptor 2/EGF Receptor Tyrosine Kinase Inhibitor Protects Cardiac Cells'. *Proceedings of the National Academy of Sciences of the United States of America* 104 (25), 10607-10612.

Speed, B., Bu, H. Z., Pool, W. F., Peng, G. W., Wu, E. Y., Patyna, S., Bello, C., and Kang, P. (2012) 'Pharmacokinetics, Distribution, and Metabolism of [14C]Sunitinib

in Rats, Monkeys, and Humans'. *Drug Metabolism and Disposition: The Biological Fate of Chemicals* 40 (3), 539-555.

Stapleton, D., Mitchelhill, K. I., Gao, G., Widmer, J., Michell, B. J., Teh, T., House, C. M., Fernandez, C. S., Cox, T., Witters, L. A., and Kemp, B. E. (1996) 'Mammalian AMP-Activated Protein Kinase Subfamily'. *The Journal of Biological Chemistry* 271 (2), 611-614.

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., and Lazar, M. A. (2001) 'The Hormone Resistin Links Obesity to Diabetes'. *Nature* 409 (6818), 307-312. 8.

Storozhuk, Y., Hopmans, S., Sanli, T., Barron, C., Tsiani, E., Cutz, J., Pond, G., Wright, J., Singh, G., and Tsakiridis, T. (2013) 'Metformin Inhibits Growth and Enhances Radiation Response of Non-Small Cell Lung Cancer (NSCLC) through ATM and AMPK'. *British Journal of Cancer* 108 (10), 2021.

Solskov, L., Løfgren, B., Kristiansen, S. B., Jessen, N., Pold, R., Nielsen, T. T., Bøtker, H. E., Schmitz, O., and Lund, S. (2008) 'Metformin Induces Cardioprotection Against ischaemia/reperfusion Injury in the Rat Heart 24 Hours After Administration'. *Basic & Clinical Pharmacology & Toxicology* 103 (1), 82-87.

Song, C. W., Lee, H., Dings, R. P., Williams, B., Powers, J., Dos Santos, T., Choi, B., and Park, H. J. (2012) 'Metformin Kills and Radiosensitizes Cancer Cells and Preferentially Kills Cancer Stem Cells'. *Scientific Reports* 2.

Soni, S. and Padwad, Y. S. (2017) 'HIF-1 in Cancer Therapy: Two Decade Long Story of a Transcription Factor'. *Acta Oncologica* 56 (4), 503-515.

Sridharan, V., Thomas, C. J., Cao, M., Melnyk, S. B., Pavliv, O., Joseph, J., Singh, S. P., Sharma, S., Moros, E. G., and Boerma, M. (2016) 'Effects of Local Irradiation Combined with Sunitinib on Early Remodeling, Mitochondria, and Oxidative Stress in the Rat Heart'. *Radiotherapy and Oncology* 119 (2), 259-264.

Stein, S. C., Woods, A., Jones, N. A., Davison, M. D., and Carling, D. (2000) 'The Regulation of AMP-Activated Protein Kinase by Phosphorylation'. *The Biochemical Journal* 345 Pt 3, 437-443.

Suematsu, N., Tsutsui, H., Wen, J., Kang, D., Ikeuchi, M., Ide, T., Hayashidani, S., Shiomi, T., Kubota, T., Hamasaki, N., and Takeshita, A. (2003) 'Oxidative Stress Mediates Tumor Necrosis Factor-Alpha-Induced Mitochondrial DNA Damage and Dysfunction in Cardiac Myocytes'*. Circulation* 107 (10), 1418-1423.

Sum, C., Webster, J., Johnson, A., Catalano, C., Cooper, B., and Taylor, R. (1992) 'The Effect of Intravenous Metformin on Glucose Metabolism during Hyperglycaemia in Type 2 Diabetes'*. Diabetic Medicine* 9 (1), 61-65.

Sun, Y., Connors, K. E., and Yang, D. (2007) 'AICAR Induces Phosphorylation of AMPK in an ATM-Dependent, LKB1-Independent Manner'*. Molecular and Cellular Biochemistry*306 (1-2), 239-245.

Sun, Q., Chen, X., Ma, J., Peng, H., Wang, F., Zha, X., Wang, Y., Jing, Y., Yang, H., Chen, R., Chang, L., Zhang, Y., Goto, J., Onda, H., Chen, T., Wang, M. R., Lu, Y., You, H., Kwiatkowski, D., and Zhang, H. (2011) 'Mammalian Target of Rapamycin Up-Regulation of Pyruvate Kinase Isoenzyme Type M2 is Critical for Aerobic Glycolysis and Tumor Growth'*. Proceedings of the National Academy of Sciences of the United States of America* 108 (10), 4129-4134.

Sun, J., Sun, Q., Brown, M. F., Dudgeon, C., Chandler, J., Xu, X., Shu, Y., Zhang, L., and Yu, J. (2012) 'The Multi-Targeted Kinase Inhibitor Sunitinib Induces Apoptosis in Colon Cancer Cells Via PUMA'*. PLoS One* 7 (8), e43158.

Sutherland, F. J. and Hearse, D. J. (2000) 'The Isolated Blood and Perfusion Fluid Perfused Heart'*. Pharmacological Research* 41 (6), 613-627.

Suwa, M., Egashira, T., Nakano, H., Sasaki, H., and Kumagai, S. (2006) 'Metformin Increases the PGC-1alpha Protein and Oxidative Enzyme Activities Possibly Via AMPK Phosphorylation in Skeletal Muscle in Vivo'*. Journal of Applied Physiology (Bethesda, Md.: 1985)* 101 (6), 1685-1692.

Takemura, Y., Osuga, Y., Yoshino, O., Hasegawa, A., Hirata, T., Hirota, Y., Nose, E., Morimoto, C., Harada, M., and Koga, K. (2007) 'Metformin Suppresses Interleukin (IL)-1β-Induced IL-8 Production, Aromatase Activation, and Proliferation

of Endometriotic Stromal Cells'. *The Journal of Clinical Endocrinology & Metabolism* 92 (8), 3213-3218.

Tang, Y., Williams, B. R., Siegel, J. J., and Amon, A. (2011) 'Identification of Aneuploidy-Selective Antiproliferation Compounds'. *Cell* 144 (4), 499-512.

Taylor, E. B., Ellingson, W. J., Lamb, J. D., Chesser, D. G., and Winder, W. W. (2005) 'Long-Chain Acyl-CoA Esters Inhibit Phosphorylation of AMP-Activated Protein Kinase at Threonine-172 by LKB1/STRAD/MO25'. *American Journal of Physiology.Endocrinology and Metabolism* 288 (6), E1055-61.

Telli, M. L., Witteles, R. M., Fisher, G. A., and Srinivas, S. (2008) 'Cardiotoxicity Associated with the Cancer Therapeutic Agent Sunitinib Malate'. *Annals of Oncology: Official Journal of the European Society for Medical Oncology*19 (9), 1613-1618.

Teng, A. C., Miyake, T., Yokoe, S., Zhang, L., Rezende, L. M.,Jr, Sharma, P., MacLennan, D. H., Liu, P. P., and Gramolini, A. O. (2015) 'Metformin Increases Degradation of Phospholamban Via Autophagy in Cardiomyocytes'. *Proceedings of the National Academy of Sciences of the United States of America* 112 (23), 7165-7170.

Terai, K., Hiramoto, Y., Masaki, M., Sugiyama, S., Kuroda, T., Hori, M., Kawase, I., and Hirota, H. (2005) 'AMP-Activated Protein Kinase Protects Cardiomyocytes Against Hypoxic Injury through Attenuation of Endoplasmic Reticulum Stress'. *Molecular and Cellular Biology* 25 (21), 9554-9575.

Teresi, R. E., Shaiu, C., Chen, C., Chatterjee, V. K., Waite, K. A., and Eng, C. (2006) 'Increased PTEN Expression due to Transcriptional Activation of PPARγ by Lovastatin and Rosiglitazone'. *International Journal of Cancer* 118 (10), 2390-2398.

Thampy, K. G. (1989) 'Formation of Malonyl Coenzyme A in Rat Heart. Identification and Purification of an Isozyme of A Carboxylase from Rat Heart'. *The Journal of Biological Chemistry* 264 (30), 17631-17634.

Thomas, G. V., Tran, C., Mellinghoff, I. K., Welsbie, D. S., Chan, E., Fueger, B., Czernin, J., and Sawyers, C. L. (2006) 'Hypoxia-Inducible Factor Determines

Sensitivity to Inhibitors of mTOR in Kidney Cancer'. *Nature Medicine* 12 (1), 122-127.

Tiainen, M., Vaahtomeri, K., Ylikorkala, A., and Makela, T. P. (2002) 'Growth Arrest by the LKB1 Tumor Suppressor: Induction of p21(WAF1/CIP1)'. *Human Molecular Genetics* 11 (13), 1497-1504.

Tian, R., Musi, N., D'Agostino, J., Hirshman, M. F., and Goodyear, L. J. (2001) 'Increased Adenosine Monophosphate-Activated Protein Kinase Activity in Rat Hearts with Pressure-Overload Hypertrophy'. *Circulation* 104 (14), 1664-1669.

Tomic, T., Botton, T., Cerezo, M., Robert, G., Luciano, F., Puissant, A., Gounon, P., Allegra, M., Bertolotto, C., and Bereder, J. (2011) 'Metformin Inhibits Melanoma Development through Autophagy and Apoptosis Mechanisms'. *Cell Death & Disease* 2 (9), e199.

Towfighi, A., Zheng, L., and Ovbiagele, B. (2009) 'Sex-Specific Trends in Midlife Coronary Heart Disease Risk and Prevalence'. *Archives of Internal Medicine* 169 (19), 1762-1766.

Towler, M. C. and Hardie, D. G. (2007) 'AMP-Activated Protein Kinase in Metabolic Control and Insulin Signaling'. *Circulation Research* 100 (3), 328-341.

Traub, M. L. (2011) 'Assessing and Treating Insulin Resistance in Women with Polycystic Ovarian Syndrome'. *World Journal of Diabetes* 2 (3), 33-40.

Tsuruta, F., Masuyama, N., and Gotoh, Y. (2002) 'The Phosphatidylinositol 3-Kinase (PI3K)-Akt Pathway Suppresses Bax Translocation to Mitochondria'. *The Journal of Biological Chemistry* 277 (16), 14040-14047.

Ueda, Y. and Richmond, A. (2006) 'NF-κB Activation in Melanoma'. *Pigment Cell Research* 19 (2), 112-124.

UK Prospective Diabetes Study (UKPDS) Group (1998) 'Effect of Intensive Blood-Glucose Control with Metformin on Complications in Overweight Patients with Type 2 Diabetes (UKPDS 34)'. *The Lancet* 352 (9131), 854-865.

Urbanek, M., Woodroffe, A., Ewens, K., Diamanti-Kandarakis, E., Legro, R., Strauss III, J., Dunaif, A., and Spielman, R. (2005) 'Candidate Gene Region for Polycystic Ovary Syndrome on Chromosome 19p13. 2'*. The Journal of Clinical Endocrinology & Metabolism* 90 (12), 6623-6629.

U.S. National Library of Medicine (n.d) *ClinicalTrials.gov.* U.S. National Institute of Health [online] available from < https://clinicaltrials.gov/ct2/results?term=metformin+and+cancer> [08/September/2015].

Varga, Z. V., Ferdinandy, P., Liaudet, L., and Pacher, P. (2015) 'Drug-Induced Mitochondrial Dysfunction and Cardiotoxicity'*. American Journal of Physiology.Heart and Circulatory Physiology* 309 (9), H1453-67.

van der Heide, R. S. and Reimer, K. A. (1996) 'Effect of Adenosine Therapy at Reperfusion on Myocardial Infarct Size in Dogs'*. Cardiovascular Research* 31 (5), 711-718.

van Haelst, P. L., Schot, B., Hoendermis, E. S., and van den Berg, M. P. (2006) 'Acute Myeloid Leukaemia as a Cause of Acute Ischaemic Heart Disease'*. Netherlands Heart Journal: Monthly Journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation* 14 (2), 62-65.

van Melle, J. P., Bot, M., de Jonge, P., de Boer, R. A., van Veldhuisen, D. J., and Whooley, M. A. (2010) 'Diabetes, Glycemic Control, and New-Onset Heart Failure in Patients with Stable Coronary Artery Disease: Data from the Heart and Soul Study'*. Diabetes Care* 33 (9), 2084-2089.

Vazquez-Martin, A., Oliveras-Ferraros, C., and Menendez, J. A. (2009) 'The Antidiabetic Drug Metformin Suppresses HER2 (erbB-2) Oncoprotein Overexpression via Inhibition of the mTOR Effector p70S6K1 in Human Breast Carcinoma Cells'*. Cell Cycle* 8 (1), 88-96.

Vazquez-Martin, A., Oliveras-Ferraros, C., Cufí, S., Martin-Castillo, B., and Menendez, J. A. (2011) 'Metformin Activates an Ataxia Telangiectasia Mutated

(ATM)/Chk2-Regulated DNA Damage-Like Response'. *Cell Cycle* 10 (9), 1499-1501.

Vecchio, S. and Protti, A. (2011) 'Metformin-Induced Lactic Acidosis: No One Left Behind'. *Critical Care* 15 (1), 107.

Vega, R. B., Huss, J. M., and Kelly, D. P. (2000) 'The Coactivator PGC-1 Cooperates with Peroxisome Proliferator-Activated Receptor Alpha in Transcriptional Control of Nuclear Genes Encoding Mitochondrial Fatty Acid Oxidation Enzymes'. *Molecular and Cellular Biology* 20 (5), 1868-1876.

Verhoeven, A. J., Woods, A., Brennan, C. H., Hawley, S. A., Hardie, D. G., Scott, J., Beri, R. K., and Carling, D. (1995) 'The AMP-activated Protein Kinase Gene is Highly Expressed in Rat Skeletal Muscle'. *The FEBS Journal* 228 (2), 236-243.

Verma, S. and McNeill, J. H. (1994) 'Metformin Improves Cardiac Function in Isolated Streptozotocin-Diabetic Rat Hearts'. *The American Journal of Physiology* 266 (2 Pt 2), H714-9.

Viana, R., Aguado, C., Esteban, I., Moreno, D., Viollet, B., Knecht, E., and Sanz, P. (2008) 'Role of AMP-Activated Protein Kinase in Autophagy and Proteasome Function'. *Biochemical and Biophysical Research Communications* 369 (3), 964-968.

Vigneau, C., Lorcy, N., Dolley-Hitze, T., Jouan, F., Arlot-Bonnemains, Y., Laguerre, B., Verhoest, G., Goujon, J. M., Belaud-Rotureau, M., and Rioux-Leclercq, N. (2013) 'All Anti-Vascular Endothelial Growth Factor Drugs can induce 'pre-Eclampsia-Like Syndrome': A RARe Study'. *Nephrology Dialysis Transplantation* 29 (2), 325-332.

Vigneri, P., Frasca, F., Sciacca, L., Pandini, G., and Vigneri, R. (2009) 'Diabetes and Cancer'. *Endocrine-Related Cancer* 16 (4), 1103-1123.

Vignozzi, L., Filippi, S., Comeglio, P., Cellai, I., Morelli, A., Rastrelli, G., Maneschi, E., Mannucci, E., and Maggi, M. (2014) 'Metformin in Vitro and in Vivo Increases Adenosine Signaling in Rabbit Corpora Cavernosa'. *The Journal of Sexual Medicine* 11 (7), 1694-1708.

Villanueva, A., Chiang, D. Y., Newell, P., Peix, J., Thung, S., Alsinet, C., Tovar, V., Roayaie, S., Minguez, B., and Sole, M. (2008) 'Pivotal Role of mTOR Signaling in Hepatocellular Carcinoma'. *Gastroenterology* 135 (6), 1972-1983. e11.

Viollet, B., Horman, S., Leclerc, J., Lantier, L., Foretz, M., Billaud, M., Giri, S., and Andreelli, F. (2010) 'AMPK Inhibition in Health and Disease'. *Critical Reviews in Biochemistry and Molecular Biology* 45 (4), 276-295.

Vogt, J., Traynor, R., and Sapkota, G. P. (2011) 'The Specificities of Small Molecule Inhibitors of the TGFss and BMP Pathways'. *Cellular Signalling* 23 (11), 1831-1842.

Vujic, I., Sanlorenzo, M., Posch, C., Esteve-Puig, R., Yen, A. J., Kwong, A., Tsumura, A., Murphy, R., Rappersberger, K., and Ortiz-Urda, S. (2015) 'Metformin and Trametinib have Synergistic Effects on Cell Viability and Tumor Growth in NRAS Mutant Cancer'. *Oncotarget* 6 (2), 969-978.

Warden, S. M., Richardson, C., O'Donnell, J.,Jr, Stapleton, D., Kemp, B. E., and Witters, L. A. (2001) 'Post-Translational Modifications of the Beta-1 Subunit of AMP-Activated Protein Kinase Affect Enzyme Activity and Cellular Localization'. *The Biochemical Journal* 354 (Pt 2), 275-283.

Wang, J., Schaner, M. E., Thomassen, S., Su, S., Piquette-Miller, M., and Giacomini, K. M. (1997) 'Functional and Molecular Characteristics of Na -Dependent Nucleoside Transporters'. *Pharmaceutical Research* 14 (11), 1524-1532.

Wang, Q. D., Pernow, J., Sjoquist, P. O., and Ryden, L. (2002) 'Pharmacological Possibilities for Protection Against Myocardial Reperfusion Injury'. *Cardiovascular Research* 55 (1), 25-37.

Wang, W., Yang, X., Lopez de Silanes, I., Carling, D., and Gorospe, M. (2003) 'Increased AMP:ATP Ratio and AMP-Activated Protein Kinase Activity during Cellular Senescence Linked to Reduced HuR Function'. *The Journal of Biological Chemistry* 278 (29), 27016-27023.

Wang, P., Yu, J., and Zhang, L. (2007) 'The Nuclear Function of p53 is Required for PUMA-Mediated Apoptosis Induced by DNA Damage'. *Proceedings of the National Academy of Sciences of the United States of America* 104 (10), 4054-4059.

Wang, Y., Qu, J., Wu, X., Hou, L., Erkkola, R., and Wang, Y. (2010) 'Different Phenotypes of Polycystic Ovary Syndrome by Rotterdam Criteria are Differently Steroidogenic but Similarly Insulin Resistant'. *Fertility and Sterility* 93 (4), 1362-1365.

Wang, E. T., Cirillo, P. M., Vittinghoff, E., Bibbins-Domingo, K., Cohn, B. A., and Cedars, M. I. (2011) 'Menstrual Irregularity and Cardiovascular Mortality'. *The Journal of Clinical Endocrinology & Metabolism* 96 (1), E114-E118.

Wang, C. H., Wey, K. C., Mo, L. R., Chang, K. K., Lin, R. C., and Kuo, J. J. (2015) 'Current Trends and Recent Advances in Diagnosis, Therapy, and Prevention of Hepatocellular Carcinoma'. *Asian Pacific Journal of Cancer Prevention : APJCP* 16 (9), 3595-3604.

Wang, X., Yang, L., Kang, L., Li, J., Yang, L., Zhang, J., Liu, J., Zhu, M., Zhang, Q., and Shen, Y. (2017) 'Metformin Attenuates Myocardial Ischemia-Reperfusion Injury via Up-Regulation of Antioxidant Enzymes'. *PloS One* 12 (8), e0182777.

Ward, J. L., Sherali, A., Mo, Z. P., and Tse, C. M. (2000) 'Kinetic and Pharmacological Properties of Cloned Human Equilibrative Nucleoside Transporters, ENT1 and ENT2, Stably Expressed in Nucleoside Transporter-Deficient PK15 Cells. Ent2 Exhibits a Low Affinity for Guanosine and Cytidine but a High Affinity for Inosine'. *The Journal of Biological Chemistry* 275 (12), 8375-8381.

Wardle, E. N. (2001) 'Nuclear Factor kappaB for the Nephrologist'. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association* 16 (9), 1764-1768.

Watanabe, C. (1918) 'Studies in the Metabolic Changes Induced by Administration of Guanidine Bases'. *J.Biol.Chem.*33, 253.

Wayman, N. S., Hattori, Y., McDonald, M. C., Mota-Filipe, H., Cuzzocrea, S., Pisano, B., Chatterjee, P. K., and Thiemermann, C. (2002) 'Ligands of the Peroxisome Proliferator-Activated Receptors (PPAR-Gamma and PPAR-Alpha) Reduce Myocardial Infarct Size'. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 16 (9), 1027-1040.

WCRF (2015), ‘Liver Cancer Statistics’, *World Cancer Research Fund international* [online] available from < http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/liver-cancer-statistics > [25th August 2015].

Webster, K. A., Discher, D. J., Kaiser, S., Hernandez, O., Sato, B., and Bishopric, N. H. (1999) 'Hypoxia-Activated Apoptosis of Cardiac Myocytes Requires Reoxygenation Or a pH Shift and is Independent of p53'*. The Journal of Clinical Investigation* 104 (3), 239-252.

Weekes, J., Hawley, S. A., Corton, J., Shugar, D., and Hardie, D. G. (1994) 'Activation of Rat Liver AMP-activated Protein Kinase by Kinase Kinase in a Purified, Reconstituted System'*. The FEBS Journal* 219 (3), 751-757.

Weiss, J. N., Korge, P., Honda, H. M., and Ping, P. (2003) 'Role of the Mitochondrial Permeability Transition in Myocardial Disease'*. Circulation Research* 93 (4), 292-301.

Weiss, R. G., Gerstenblith, G., and Bottomley, P. A. (2005) 'ATP Flux through Creatine Kinase in the Normal, Stressed, and Failing Human Heart'*. Proceedings of the National Academy of Sciences of the United States of America* 102 (3), 808-813.

Whittington, H. J., Hall, A. R., McLaughlin, C. P., Hausenloy, D. J., Yellon, D. M., and Mocanu, M. M. (2013) 'Chronic Metformin Associated Cardioprotection Against Infarction: Not just a Glucose Lowering Phenomenon'*. Cardiovascular Drugs and Therapy* 27 (1), 5-16.

Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999) 'Mitogen-Activated Protein Kinase: Conservation of a Three-Kinase Module from Yeast to Human'*. Physiological Reviews* 79 (1), 143-180.

Wiernsperger, N. (1999) 'Membrane Physiology as a Basis for the Cellular Effects of Metformin in Insulin Resistance and Diabetes'*. Measurements*1000, 7.

Wilcock, C., Wyre, N., and Bailey, C. (1991) 'Subcellular Distribution of Metformin in Rat Liver'*. Journal of Pharmacy and Pharmacology* 43 (6), 442-444.

Wild, S., Pierpoint, T., McKeigue, P., and Jacobs, H. (2000) 'Cardiovascular Disease in Women with Polycystic Ovary Syndrome at long-term follow-up: A Retrospective Cohort Study'. *Clinical Endocrinology* 52 (5), 595-600.

Will, Y., Dykens, J. A., Nadanaciva, S., Hirakawa, B., Jamieson, J., Marroquin, L. D., Hynes, J., Patyna, S., and Jessen, B. A. (2008) 'Effect of the Multitargeted Tyrosine Kinase Inhibitors Imatinib, Dasatinib, Sunitinib, and Sorafenib on Mitochondrial Function in Isolated Rat Heart Mitochondria and H9c2 Cells'. *Toxicological Sciences: An Official Journal of the Society of Toxicology* 106 (1), 153-161.

Willows, R., Sanders, M. J., Xiao, B., Patel, B. R., Martin, S. R., Read, J., Wilson, J. R., Hubbard, J., Gamblin, S. J., and Carling, D. (2017) 'Phosphorylation of AMPK by Upstream Kinases is Required for Activity in Mammalian Cells'. *The Biochemical Journal* 474 (17), 3059-3073.

Wong, K. A. and Lodish, H. F. (2006) 'A Revised Model for AMP-Activated Protein Kinase Structure: The Alpha-Subunit Binds to both the Beta- and Gamma-Subunits although there is no Direct Binding between the Beta- and Gamma-Subunits'. *The Journal of Biological Chemistry* 281 (47), 36434-36442.

Wood, A. J., Bailey, C. J., and Turner, R. C. (1996) 'Metformin'. *New England Journal of Medicine* 334 (9), 574-579.

World Health Organisation (2017) 'Cardiovascular Diseases (CVDs)'. *World Health Organisation* [online] available from <http://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)> [26th June 2018].

Wouters, B. G., van den Beucken, T., Magagnin, M. G., Koritzinsky, M., Fels, D., and Koumenis, C. (eds.) (2005) *Seminars in Cell & Developmental Biology*. 'Control of the Hypoxic Response through Regulation of mRNA Translation': Elsevier.

Xavier, D., Amaral, L., Gomes, M., Rocha, M., Campos, P., Cota, B., Tafuri, L., Paiva, A., Silva, J., and Andrade, S. (2010) 'Metformin Inhibits Inflammatory Angiogenesis in a Murine Sponge Model'. *Biomedicine & Pharmacotherapy* 64 (3), 220-225.

Xu, Q., Simpson, S. E., Scialla, T. J., Bagg, A., and Carroll, M. (2003) 'Survival of Acute Myeloid Leukemia Cells Requires PI3 Kinase Activation'. *Blood* 102 (3), 972-980.

Xu, Z., Ji, X., and Boysen, P. G. (2004) 'Exogenous Nitric Oxide Generates ROS and Induces Cardioprotection: Involvement of PKG, Mitochondrial KATP Channels, and ERK'. *American Journal of Physiology. Heart and Circulatory Physiology* 286 (4), H1433-40.

Xu, X., Lu, Z., Fassett, J., Zhang, P., Hu, X., Liu, X., Kwak, D., Li, J., Zhu, G., Tao, Y., Hou, M., Wang, H., Guo, H., Viollet, B., McFalls, E. O., Bache, R. J., and Chen, Y. (2014) 'Metformin Protects Against Systolic Overload-Induced Heart Failure Independent of AMP-Activated Protein Kinase alpha2'. *Hypertension (Dallas, Tex.: 1979)* 63 (4), 723-728.

Yamaguchi, H. and Hong-Gang, W. (2001) 'The Protein Kinase PKB/Akt Regulates Cell Survival and Apoptosis by Inhibiting Bax Conformational Change'. *Oncogene* 20 (53), 7779.

Yang, J. and Holman, G. D. (2006) 'Long-Term Metformin Treatment Stimulates Cardiomyocyte Glucose Transport through an AMP-Activated Protein Kinase-Dependent Reduction in GLUT4 Endocytosis'. *Endocrinology* 147 (6), 2728-2736.

Yang, Z. H. and Peng, X. D. (2010) 'Insulin Resistance and Heart Injury in Rats with Insulin Resistance or Type 2 Diabetes Mellitus'. *Acta Cardiologica* 65 (3), 329-335.

Yang, X., Krieg, T., Cui, L., Downey, J. M., and Cohen, M. V. (2004) 'NECA and Bradykinin at Reperfusion Reduce Infarction in Rabbit Hearts by Signaling through PI3K, ERK, and NO'. *Journal of Molecular and Cellular Cardiology* 36 (3), 411-421.

Yang, P. M., Liu, Y. L., Lin, Y. C., Shun, C. T., Wu, M. S., and Chen, C. C. (2010) 'Inhibition of Autophagy Enhances Anticancer Effects of Atorvastatin in Digestive Malignancies'. *Cancer Research* 70 (19), 7699-7709.

Yap, Y. G. and Camm, A. J. (2003) 'Drug Induced QT Prolongation and Torsades De Pointes'. *Heart (British Cardiac Society)* 89 (11), 1363-1372.

Yavari, A., Stocker, C. J., Ghaffari, S., Wargent, E. T., Steeples, V., Czibik, G., Pinter, K., Bellahcene, M., Woods, A., and De Morentin, Pablo B Martínez (2016) 'Chronic Activation of γ2 AMPK Induces Obesity and Reduces β Cell Function'. *Cell Metabolism* 23 (5), 821-836.

Yellon, D. M. and Downey, J. M. (2003) 'Preconditioning the Myocardium: From Cellular Physiology to Clinical Cardiology'. *Physiological Reviews* 83 (4), 1113-1151.

Yin, M., van der Horst, I. C., van Melle, J. P., Qian, C., van Gilst, W. H., Sillje, H. H., and de Boer, R. A. (2011) 'Metformin Improves Cardiac Function in a Nondiabetic Rat Model of Post-MI Heart Failure'. *American Journal of Physiology.Heart and Circulatory Physiology* 301 (2), H459-68.

Yoshida, T., Mett, I., Bhunia, A. K., Bowman, J., Perez, M., Zhang, L., Gandjeva, A., Zhen, L., Chukwueke, U., and Mao, T. (2010) 'Rtp801, a Suppressor of mTOR Signaling, is an Essential Mediator of Cigarette Smoke-Induced Pulmonary Injury and Emphysema'. *Nature Medicine* 16 (7), 767-773.

Yoshida, S., Hong, S., Suzuki, T., Nada, S., Mannan, A. M., Wang, J., Okada, M., Guan, K. L., and Inoki, K. (2011) 'Redox Regulates Mammalian Target of Rapamycin Complex 1 (mTORC1) Activity by Modulating the TSC1/TSC2-Rheb GTPase Pathway'. *The Journal of Biological Chemistry* 286 (37), 32651-32660.

Ytrehus, K. (2000) 'The Ischemic heart—experimental Models'. *Pharmacological Research* 42 (3), 193-203.

Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., and Vogelstein, B. (2001) 'PUMA Induces the Rapid Apoptosis of Colorectal Cancer Cells'. *Molecular Cell* 7 (3), 673-682.

Yu, J., Wang, Z., Kinzler, K. W., Vogelstein, B., and Zhang, L. (2003) 'PUMA Mediates the Apoptotic Response to p53 in Colorectal Cancer Cells'. *Proceedings of the National Academy of Sciences of the United States of America* 100 (4), 1931-1936.

Yu, J. and Zhang, L. (2004) 'Apoptosis in Human Cancer Cells'. *Current Opinion in Oncology* 16 (1), 19-24.

Yu, H., Steeghs, N., Kloth, J. S., Wit, D., Hasselt, J., Erp, N. P., Beijnen, J. H., Schellens, J. H., Mathijssen, R. H., and Huitema, A. D. (2015) 'Integrated semi-physiological Pharmacokinetic Model for both Sunitinib and its Active Metabolite SU12662'. *British Journal of Clinical Pharmacology* 79 (5), 809-819.

Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z. W., Karin, M., and Shoelson, S. E. (2001) 'Reversal of Obesity- and Diet-Induced Insulin Resistance with Salicylates Or Targeted Disruption of Ikkbeta'. *Science (New York, N.Y.)* 293 (5535), 1673-1677.

Yuan, J., Lipinski, M., and Degterev, A. (2003) 'Diversity in the Mechanisms of Neuronal Cell Death'. *Neuron* 40 (2), 401-413.

Yuan, L., Ziegler, R., and Hamann, A. (2003) 'Metformin Modulates Insulin Post-Receptor Signaling Transduction in Chronically Insulin-Treated Heps G2 Cells'. *Acta Pharmacologica Sinica* 24 (1), 55-60.

Yuan, T. and Cantley, L. (2008) 'PI3K Pathway Alterations in Cancer: Variations on a Theme'. *Oncogene* 27 (41), 5497-5510.

Yue, T. L., Bao, W., Jucker, B. M., Gu, J. L., Romanic, A. M., Brown, P. J., Cui, J., Thudium, D. T., Boyce, R., Burns-Kurtis, C. L., Mirabile, R. C., Aravindhan, K., and Ohlstein, E. H. (2003) 'Activation of Peroxisome Proliferator-Activated Receptor-Alpha Protects the Heart from ischemia/reperfusion Injury'. *Circulation* 108 (19), 2393-2399.

Zakikhani, M., Dowling, R., Fantus, I. G., Sonenberg, N., and Pollak, M. (2006) 'Metformin is an AMP Kinase-Dependent Growth Inhibitor for Breast Cancer Cells'. *Cancer Research* 66 (21), 10269-10273.

Zamorano, J. L., Lancellotti, P., Rodriguez Muñoz, D., Aboyans, V., Asteggiano, R., Galderisi, M., Habib, G., Lenihan, D. J., Lip, G. Y., and Lyon, A. R. (2016) '2016 ESC Position Paper on Cancer Treatments and Cardiovascular Toxicity Developed

Under the Auspices of the ESC Committee for Practice Guidelines: The Task Force for Cancer Treatments and Cardiovascular Toxicity of the European Society of Cardiology (ESC)'. *European Heart Journal* 37 (36), 2768-2801.

Zhang, L., He, H., and Balschi, J. A. (2007) 'Metformin and Phenformin Activate AMP-Activated Protein Kinase in the Heart by Increasing Cytosolic AMP Concentration'. *American Journal of Physiology.Heart and Circulatory Physiology* 293 (1), H457-66.

Zhang, J., Yang, P. L., and Gray, N. S. (2009) 'Targeting Cancer with Small Molecule Kinase Inhibitors'. *Nature Reviews Cancer* 9 (1), 28-39.

Zhang, L. and Yu, J. (2013) 'Role of Apoptosis in Colon Cancer Biology, Therapy, and Prevention'. *Current Colorectal Cancer Reports* 9 (4), 331-340.

Zhao, T., Zhao, W., Chen, Y., Ahokas, R. A., and Sun, Y. (2010) 'Vascular Endothelial Growth Factor (VEGF)-A: Role on Cardiac Angiogenesis Following Myocardial Infarction'. *Microvascular Research* 80 (2), 188-194.

Zheng, L., Yang, W., Wu, F., Wang, C., Yu, L., Tang, L., Qiu, B., Li, Y., Guo, L., Wu, M., Feng, G., Zou, D., and Wang, H. (2013) 'Prognostic Significance of AMPK Activation and Therapeutic Effects of Metformin in Hepatocellular Carcinoma'. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 19 (19), 5372-5380.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J., and Moller, D. E. (2001) 'Role of AMP-Activated Protein Kinase in Mechanism of Metformin Action'. *The Journal of Clinical Investigation* 108 (8), 1167-1174.

Zou, M. H., Kirkpatrick, S. S., Davis, B. J., Nelson, J. S., Wiles, W. G.,4th, Schlattner, U., Neumann, D., Brownlee, M., Freeman, M. B., and Goldman, M. H. (2004) 'Activation of the AMP-Activated Protein Kinase by the Anti-Diabetic Drug Metformin in Vivo. Role of Mitochondrial Reactive Nitrogen Species'. *The Journal of Biological Chemistry* 279 (42), 43940-43951.

Zhou, K., Bellenguez, C., Spencer, C., Bennett, A., Coleman, R., Tavendale, R., Hawley, S., Donnelly, L., Schofield, C., and Groves, C. 'GoD-ARTS and UKPDS Diabetes Pharmacogenetics Study Group; Wellcome Trust Case Control Consortium 2; MAGIC Investigators (2011). Common Variants Near ATM are Associated with Glycemic Response to Metformin in Type 2 Diabetes'*. Nat.Genet* 43, 117-120.

Zimmermann, S. and Moelling, K. (1999) 'Phosphorylation and Regulation of Raf by Akt (Protein Kinase B)'*. Science (New York, N.Y.)* 286 (5445), 1741-1744.